SYNTHETIC POLYNUCLEOTIDES AND INTERFERON INDUCTION

By

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1973.

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DEDICATION

To My Parents.

I solemnly dedicate this thesis to the memory of my father late Chief Esarun Atikekere Folayan. Dad, I thank you for your unceasing ambition and determination to educate me to this level through thick and thin. What a pity your sun was set while it was still day, with me five thousand miles away here in England and not around to bury you. I thank my Parents-in-law, Revd. Canon and Mrs. R. A. Rotimi for giving you a decent burial on my behalf and for encouraging me to complete this course.

I dedicate this thesis to you too, my mother, Mrs. Ayanponmle Aweke Folayan. I appreciate all your endeavour to get me this far in my academic career. You have the future to look forward to as tomorrow is just the beginning of the rest of our lives.

May the soul of my Dad rest in peace and may God grant my Mum long life to reap the fruits of her labour.

C O N T E N T S.

ACKNOWLEDGEMENTS.

The work reported in this thesis is the original work of the author, except where specific acknowledgement is made, and has not been submitted for a degree at any other University.

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ABBREVIATIONS.

Most of the abbreviations used in this thesis are as in Biochim. Biophys. Acta. The following are for further guide.

CMP	-	Cytidine Monophosphate
AMP	-	Adenosine Monophosphate
UMP	-	Uridine Monophosphate
x^5C	-	5-substituted cytidine, where x may be F, Cl, Br, I, Me_2N, OH and C could be CH for uridine.
Me_2N	-	dimethylamine group.
x^8A	-	8-substituted adenosine, where x may be Br, O or NH_2.
PRNase	-	Pancreatic ribonuclease.
PNPase	-	Polynucleotide phosphorylase.
Poly(P-Q)	-	Copolymers of P and Q nucleotides.
Poly(P).poly(Q)	-	Hybrids of poly(P) and poly(Q).

SUMMARY.

Modified cytidylic and adenylic acids have been synthesised and studied as possible inducers of interferon when hybridised with the appropriate polynucleotides.

The report in this thesis describes:

a) The synthesis and characterisation of poly (5-fluoro-cytidylic acid) to complete the series of 5-halo substituted cytidylic acids. Poly(I).poly($f1^5C$) was interferon inducing.

b) The synthesis and characterisation of poly (5-dimethyl-aminocytidylic acid) in which a substituent other than the halogens was inserted into the 5-position of cytidine. The polymer formed a 1:1 hybrid with poly(I) and the hybrid was a good inducer of interferon.

c) The synthesis and characterisation of poly (8-oxyadenylic acid) which was one of the first 8-substituted homopolymers of adenosine.

INTRODUCTION.

Synthetic Polynucleotides.

There are three main methods for the synthesis of polynucleotides.

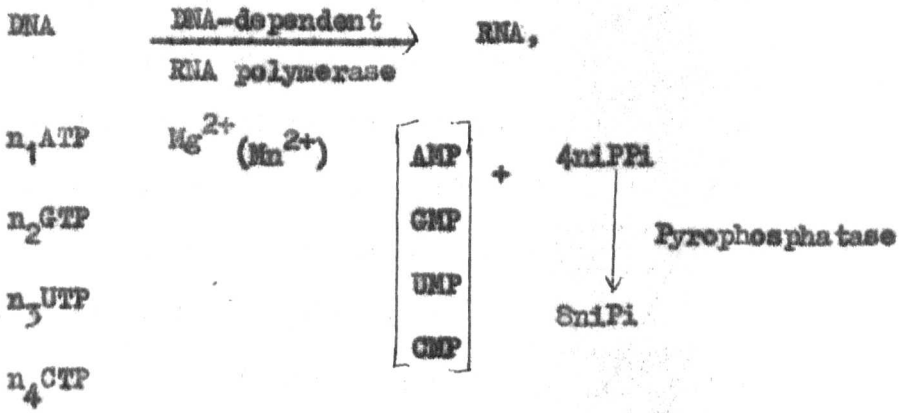
Polynucleotide phosphorylase method:- PNPase catalyses the reversible polymerisation of ribonucleoside diphosphates with a release of inorganic phosphate as shown by Godefroy et al.[6]

$$R(pN)n + Pi \underset{}{\overset{M^{2+}}{\rightleftharpoons}} R_{OH} + ppN$$

The best conditions for this enzyme's activity are when R(pN)n is a polynucleotide with a 3'-terminal hydroxyl group and at least three nucleotide units long and when R_{OH} is a polynucleotide with a 3'-terminal hydroxyl group (now serving as primer) and ppN is a ribonucleoside 5'-pyrophosphate; M^{2+} being a divalent cation like magnessium and manganous. This enzyme synthesises polymers either by elongation of primer R_{OH} or direct from the nucleoside diphosphates if no primer is present (de novo synthesis). The enzyme is capable of polymerising mixed nucleotides yielding random coils[7]. It is capable of use on a wide range of substrates. PNpase is also involved in genetic code[8] and the study of the structure of tRNA[9,10]. It is advantageous that this enzyme occurs in almost all known bacteria and it has been found even in some plant tissues[11]. Some animal tissues are found to contain this enzyme too e.g. guinea pig liver[12]. PNpase, especially from E. coli is stable up to 55-60°C for ten minutes[13] and functions between pH 7 and 10.5 although optimum at pH 8 - 9.5[14]. The enzyme has very little or no specificity for the substrate or metallic cofactor, although it does have strict restriction for the conformation of the nucleoside diphosphates and it is inhibited by calcium[15]. The kinetics of polymerisation by

PNpase of E. coli and M. lutens have been studied to a great extent[16,17] and a lag phase has been observed, in some cases (see figure 1). This lag phase can be suppressed by the addition of suitable primer[6,18]. This activates the enzyme and polymerisation may start immediately. This enzyme is ideally suited for the in vitro synthesis of homopolynucleotides.

RNA polymerase method:- RNA can be synthesized from nucleoside triphosphate by DNA-dependent RNA polymerase[1] as shown below[2].



The action of this enzyme has been studied by the use of antibiotic drugs which inhibit the activity of RNA polymerase, thus causing the RNA synthesis to be subsequently blocked in bacteria[3,4]. Although one could expect some differences between bacterial synthesis and in vitro synthesis, the basic activity of RNA polymerase is the same as illustrated by Khorana et al.[5]

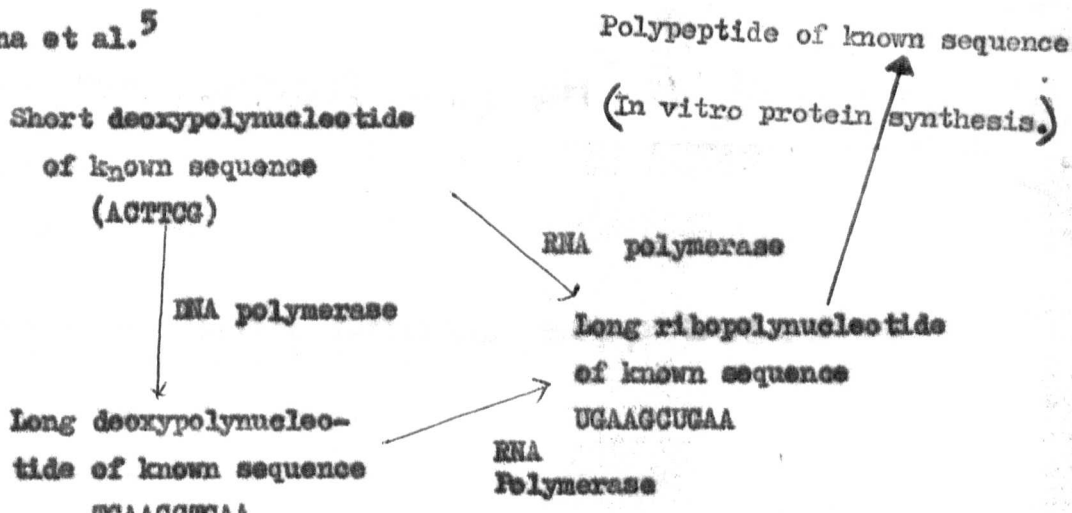
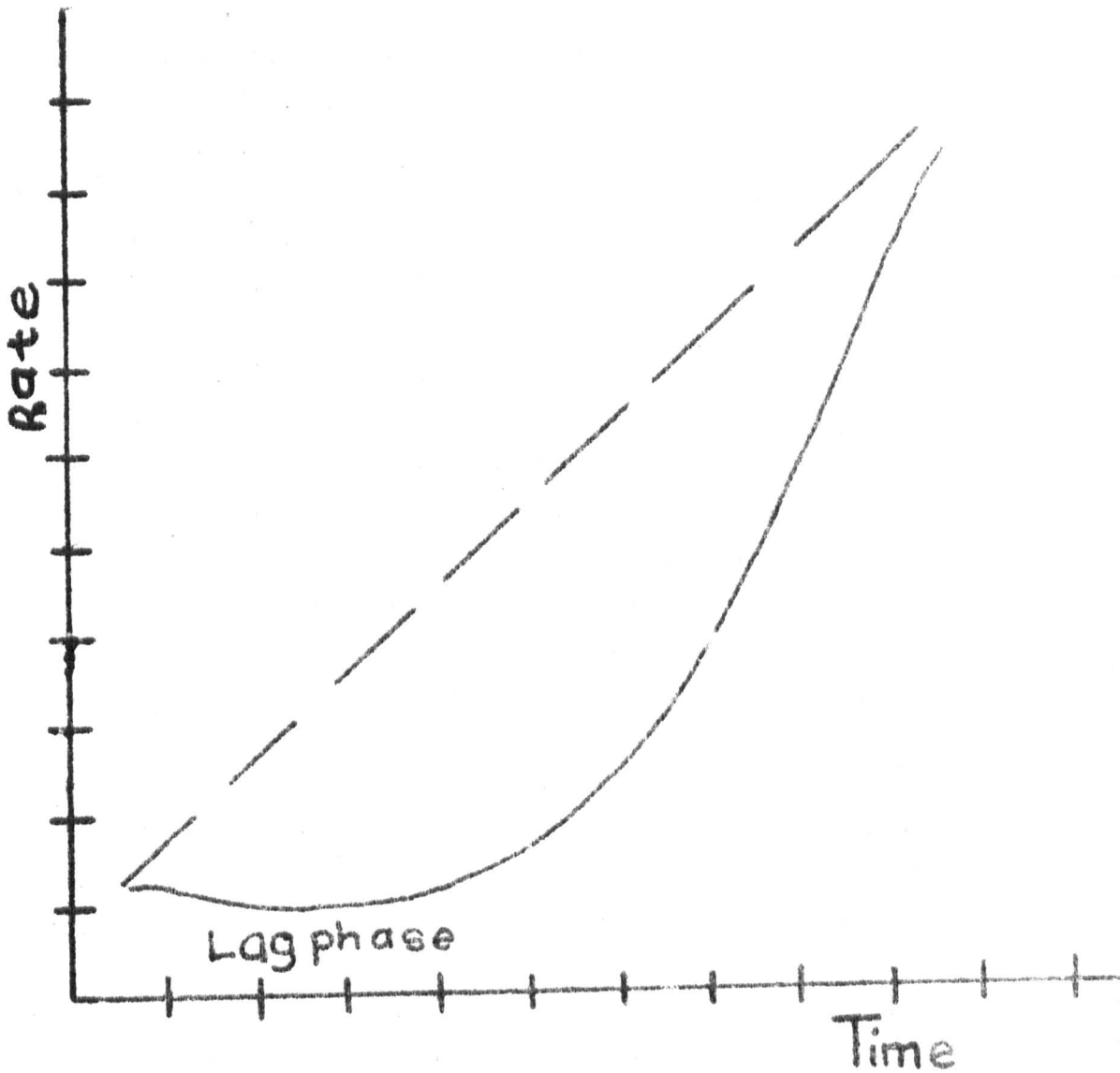


Fig. 1

Lag phase and lag phase repression in PNPase action.



~~~~ without lag phase repression.

- - - - - - - - Lag phase suppressed
~~~~

The mechanism of RNA polymerase-catalysed reaction involves association of the DNA template with the polymerase; initiation of chain; chain elongation; and chain termination by liberation of the newly synthesised RNA and enzyme from its DNA template. In vivo, only one strand of DNA is copied to give a product with a complementary base sequence. This is because only one DNA strand functions as a template. Chain elongation is in the 5' to 3' direction. E. coli. polymerase reveals the existence of five separable subunits in the enzyme ($\alpha_2\beta'\beta\omega\sigma$)[265]. β' subunit is required for binding RNA polymerase to the DNA template, β is the site of interaction of rifamycin (an antibiotic that inhibits initiation of RNA synthesis); σ is required for the correct initiation of RNA synthesis at a specific site of the DNA template.

There are some problems in the use of this enzyme. One has to use nucleoside triphosphate and not diphosphate. Also, a template is always required. The synthesis of the triphosphate and the template is sometimes a major task in itself.

Chemical synthesis:- The basic aim of chemical synthesis and its advantage is the possibility of step-by-step synthesis of polynucleotides of defined sequences. Two approaches have been developed; (a) building up a polynucleotide to a longer polynucleotide chain (b) making different blocks of polynucleotides and linking them together[19-21]. However, there are some problems involved; (1) the phosphomonoester group on the mononucleotide has to be activated to cause phosphorylation of the hydroxyl group of another nucleoside, or nucleotide; (2) the hydroxyl and amino groups of nucleosides

have to be protected[5] but solutions to these problems have been developed, as shown in scheme 2[22]. The polymers, after they have been made, have to be characterised to obtain their actual sequence. This also, has been overcome and methods have been developed to synthesize all the 64 trinucleotides that can be obtained from the four mononucleotides without any ambiguity[23].

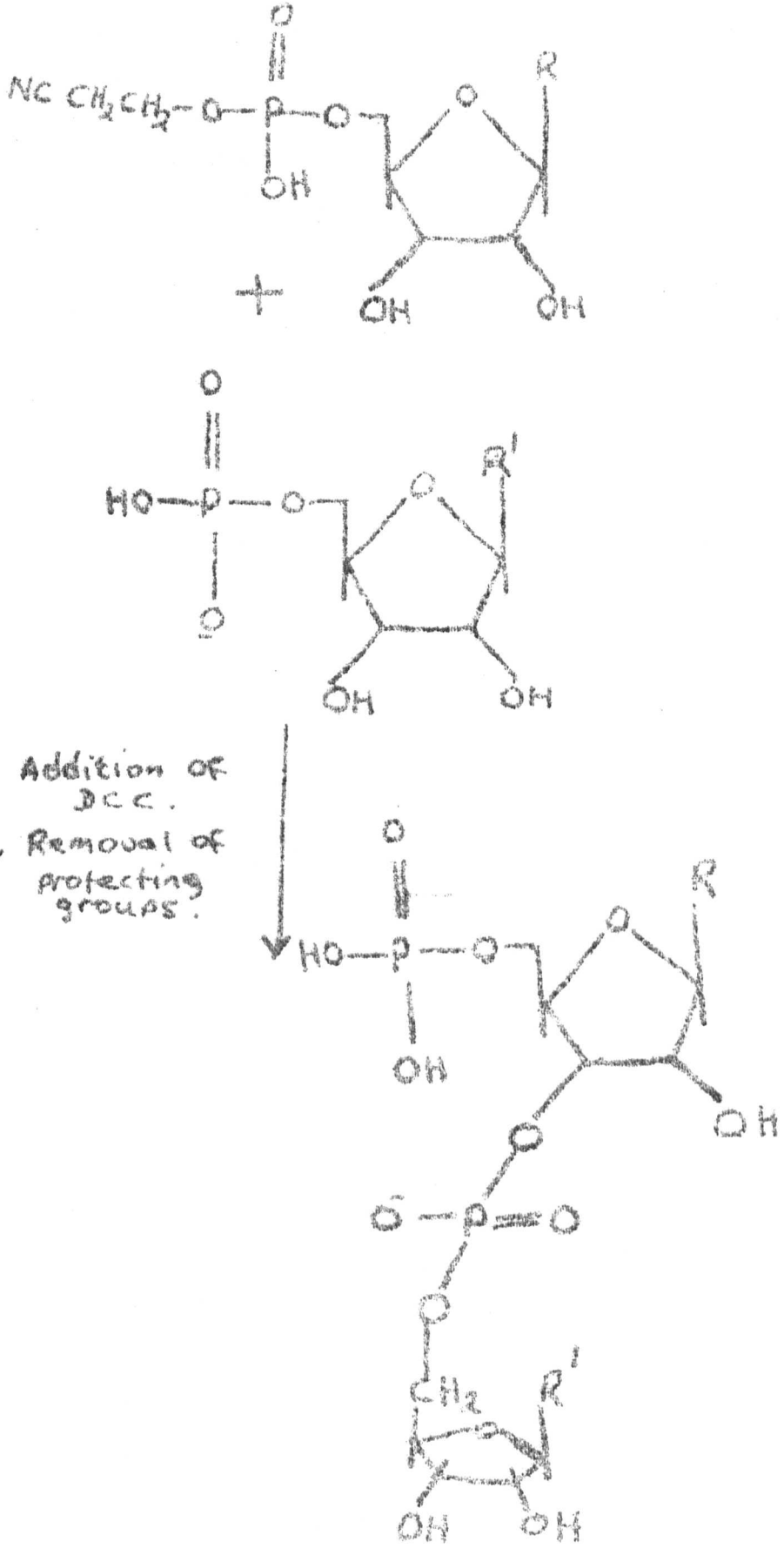
In this project polymers were synthesised by the use of PNpase as reactions were carried out through the diphosphate route. It has also been possible to work on very small scale (20 mM) nucleotide, by using PNpase method since only one sequence is expected in each case and there is no need for sequence characterisation.

Structure of polynucleotides:

A great deal of work has been done to elucidate the structure of polynucleotides using ultraviolet spectroscopy[148], optical rotatory dispersion[24], circular dichroism[25], x-ray diffraction[26], calorimetry[27] and nuclear magnetic resonance[28]. All these methods lead to similar results. For example, there is a common observation that poly(A) and its oligomers exist as single-stranded helices with partially stacked bases at neutral pH. It is commonly found too, that at acid pH poly(A) and the higher oligomers form double-stranded hydrogen-bonded helices as the pH goes below the pKa of the nucleotide concerned. It has been shown that at pH 8.5 and at low ionic concentration, the structure is not affected by a variation of the ionic concentration[29], but the structure is destroyed by adding dioxan[30]. Poly(C) also exists as single stranded at neutral pH and adopts the double-stranded helix at pH below the pKa of poly(C)[29]. There are, however, some differences between

SCHEME 2.

CHEMICAL SYNTHESIS OF POLYNUCLEOTIDES.[21]



nucleic acids as one might expect[31]. In the pH titration profiles, two breaks are observed in the titration of poly(A), one at pH 5.8 and the other at pH 3.4[32]. Poly(C) shows only one transition near the pKa[33]. These double stranded helices at acid pH's have been confirmed by the hyperchromic rise in absorbance when heated to the melting temperature at which the two strands fall apart[148].

Hybridisation of polynucleotides:- Polynucleotides have been observed to form helices with each other at neutral pH apart from the observed phenomenon of helix formation at acid pH between strands of the same polymer as mentioned above. Poly(A) and poly(U) have been observed to hybridise between each other in the ratio of one poly(A) to two poly(U), thus resulting in triple stranded helices of poly(A).poly(U)$_2$ at neutral pH[34]. This will not be expected to occur at acid pH since poly(A) will have assumed the double helix with itself and will not be available for hybridisation with poly(U). Poly(C) hybridises with poly(I) in a ratio of 1:1.

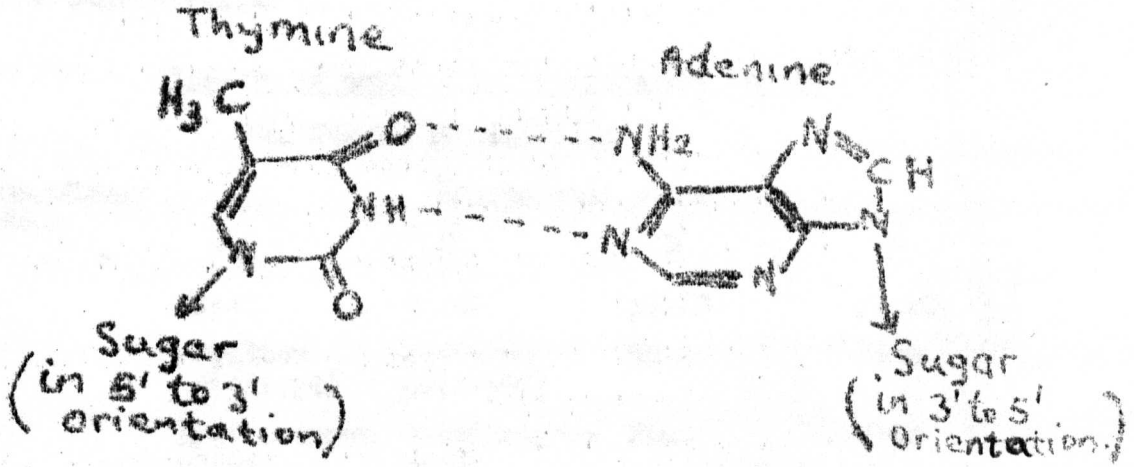
Hybridisation occurs as a result of base pairing through hydrogen bonds. The arrangement of the strands is in the antiparallel fashion, which is 5' to 3' orientation in one strand and 3' to 5' in the other (see scheme 3)[265].

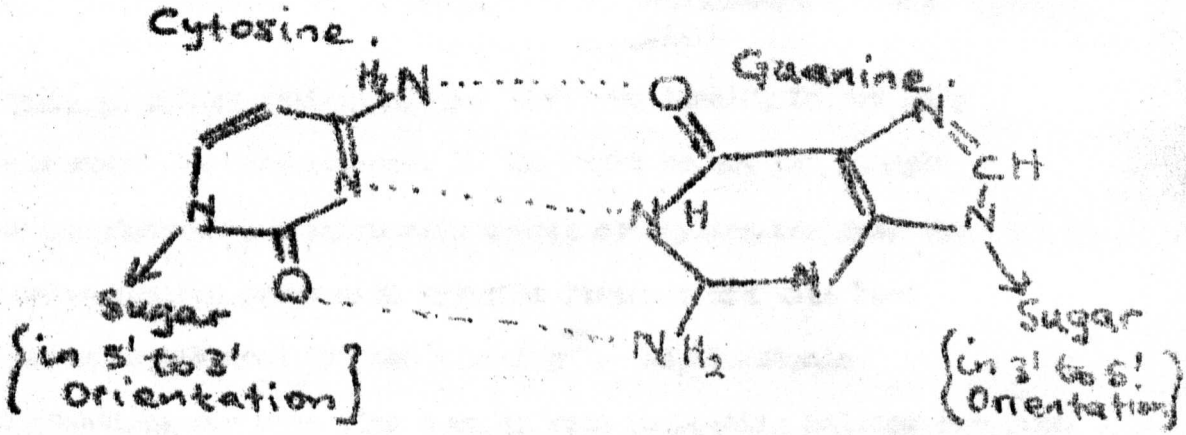
The summary of possible events is given in the table below. All these can be controlled by choosing the right salt concentration and correct concentration of polymers. Mixing equimolar solutions yields 1:1 hybrid in 0.1M sodium ion concentration[35]. At 0.6M sodium ion concentration and at a solution of two poly(dI) to one poly(dC), there is a formation of the type

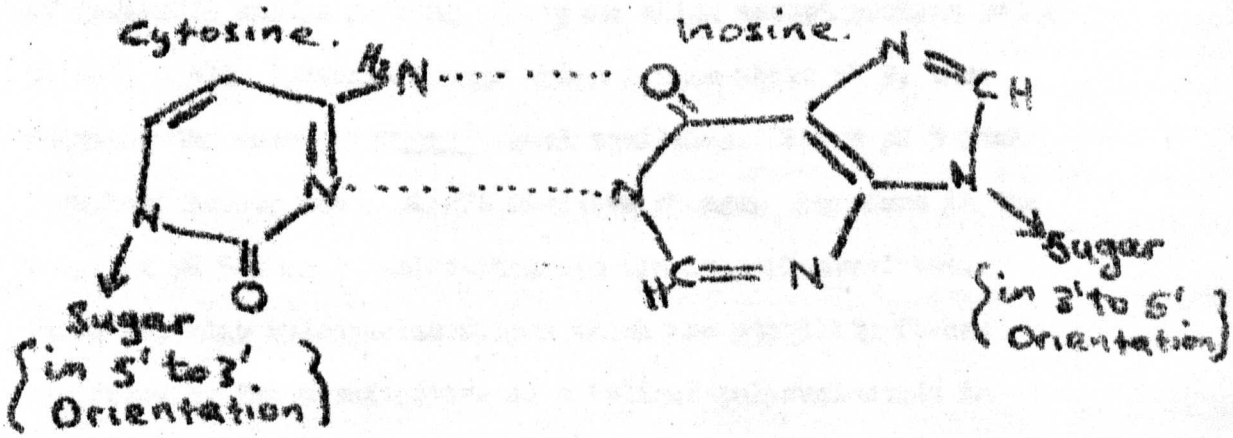
$$dI + dI : dC \rightleftharpoons dI\,(dI : dC)$$ [36].

SCHEME 5.

Base pairs in polynucleotides; arrangement is antiparallel.[265]







From the method of continuous variations[259], it has been shown that the double stranded helix is first formed followed by the triple stranded helix formation. This reaction also does not occur at below 0.5M ionic concentration or higher than 0.7M ionic concentration[37].

Summary of coil-helix interactions in the IC homopolymer pairs[37].

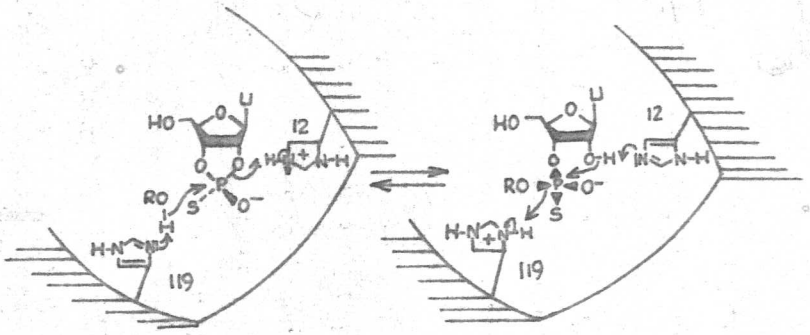
Homopolymer added.	Homopolymer pairs			
	1	2	3	4
	dI:rC	dI:dC	rI:dC	rI:rC
dI	addition dI(dI:rC)	addition dI(dI:dC)	None	None
rI	displacement rI:rC	displacement rI:dC	None	None
dC	displacement dI:dC	None	None	None
rC	None	None	displacement rI:rC	None

Forces affecting conformation:- Hydrogen bonding forces help to arrange the base residues in the right order, for example, the adenine residue pairs with uracil or thymine residue; the guanine residue pairs with cytosine residue; and this base pairing is enhanced by base stacking[38]. Dipole-dipole interactions may also play a major role in holding helices together. There are also, electrostatic forces attributable to ionisation of ionisable groups or ring nitrogens which accept protons below pH 4-5; enolic hydroxyl groups which ionise above pH 9, thus shifting the keto $\rightleftharpoons$ enol equilibra. Above pH 3 each phosphate moiety has a single negative change, therefore in the range of pH 5-9 most nucleotides are aromatic electrolytes. There are also hydrophobic forces which are stability forces conferred by the architecture of a helical polynucleotide in

which the polar phosphate groups are on the surface in an aqueous environment while the heterocyclic base rings are directed towards the interior where solvation is diminished. These forces relate to the effects of water on the polynucleotide solute and act to minimise the exposed surface of a polynucleotide so that it causes the least possible interference with the structure of the surrounding water. These forces enhance the conformation in both double helical and single stranded nucleic acids. Other forces but not as important as those mentioned above are strain energies, configurational and solvent entropy[38-40].

Nucleotides and Ribonucleases:- PRNase is known to hydrolyse RNA by a two-step mechanism; a transesterification step followed by the hydrolysis of the intermediate 2', 3'-cyclic monophosphate ester[41]. But resistance to RNase hydrolysis is important for stability of polynucleotide in a biological medium as will be seen later on. Various mechanisms postulated all point to "inline" and "adjacent" approach of the incoming group[42] as illustrated in schemes 4 and 5 below. Adjacent mechanism occurs when the same group acts first as a general base to the incoming nucleophile and then as a general acid to the outgoing group[43]. In-line mechanism implies a considerable movement of either the catalytic group or the substrate. It has also been suggested that the two steps may have different mechanisms as the hydroxyl of a nucleoside is obviously not necessarily equivalent to a water molecule[44]. There were several suggested explanations for in-line and adjacent mechanisms but all end in no clear conclusions[42-52]. Conclusive evidence for in-line mechanism was obtained from the work of Eckstein[53] on the diastereoisomers of uridine 2', 3'-cyclic phosphorothiate[54]. Pseudorotation has been used to explain the hydrolysis of phosphate esters in early

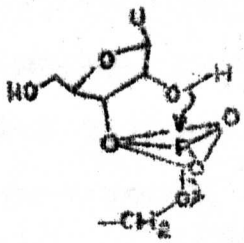
SCHEME 4.



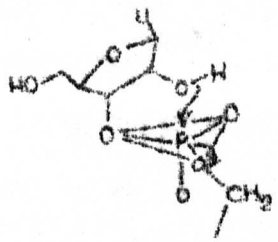
MECHANISM OF TRANSESTERIFICATION BY PANCREATIC RIBONUCLEASE.

SCHEME 5

FIRST STEP

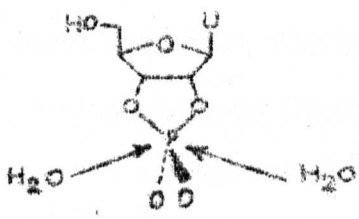


IN-LINE



ADJACENT

SECOND STEP

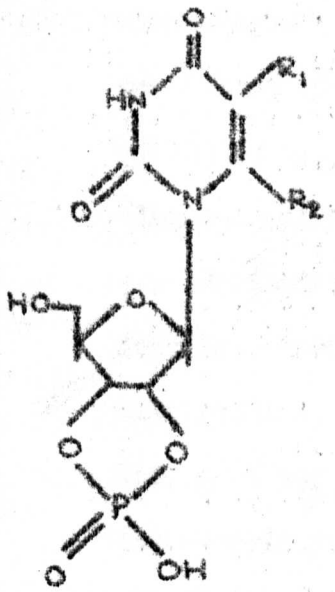


IN-LINE ADJACENT

THE IN-LINE AND ADJACENT MECHANISMS FOR THE TWO STEPS OF RIBONUCLEASE ACTION. IN THE ADJACENT MECHANISMS, THE MOLECULE WOULD UNDERGO A PSEUDOROTATION BEFORE PRODUCT FORMATION.

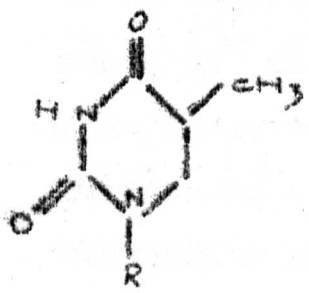
works[46-68]. Eckstein's hydrolysis was done in highly basic medium and the polar groups of the molecule would occupy apical positions of the trigonal bipyramid. Under these conditions pseudorotation would be inhibited[42] and ring closure would be in-line[43,47,55]. The results led to the conclusion that ring opening was in-line.

From the technique of stopped flow, two distinct relaxation processes have been observed[69-71]. This has been explained to be due to an initial association-dissociation of enzyme and substrate, followed by an isomerisation of the enzyme-substrate complex[72]. Because of the numerous questions that remained to be answered, a large number of 2', 3'-cyclic phosphate derivatives of pyrimidine ribonucleosides and their behaviours towards PRNase and RNase T2 have been studied[73]. The attempt was to obtain additional information on the substrate specificity and the mechanism of action of PRNase as a base specific enzyme[74,75]. Several 2', 3'-cyclic phosphates of base-substituted pyrimidine ribonucleosides and other related compounds were synthesised (scheme 6) and their behaviour towards both enzymes studied. The conclusion of course was only quantitative but very important. With the use of modified substrates, an inhibitory effect of the N_3-substituent has been proved[74]. Similarly, a carbodiimide derivative on the N_3 showed the reduction of PRNase hydrolysis of uridine[76]. The most significant point is that a number of PRNase degradations were performed on substrates IIIa - IIIf and XVI. It was found that substrates IIIa - IIIf, were good substrates for PRNase and RNase T2, substrates VI, X and XVI were good substrates to PRNase but not very good for RNase T2. Substrate IIIi was a poor substrate to both PRNase and RNase T2. These results show that

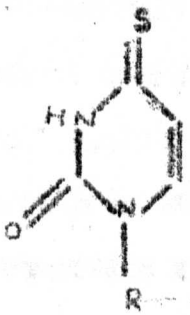


III

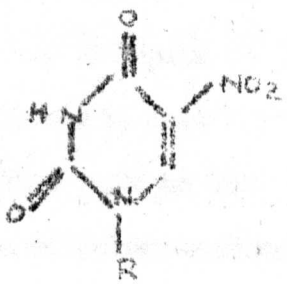
	R_1	R_2
a	Cl	H
b	F	H
c	NH_2	H
d	$(CH_3)_2N$	H
e	OH	H
f	C_2H_5	H
g	H	COOH



VI

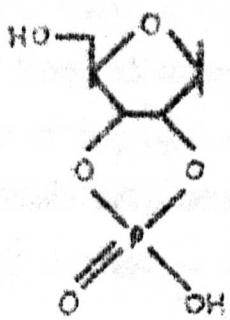


X



XVI

R =



Scheme 6.

the substituent at position 5 did not exert any significant effect on the enzymatic hydrolysis of uridine 2',3'-cyclic monophosphate. It was claimed that this observation was consistent with earlier results[74] and that with time, in the series of compounds examined[73], the substitution with either electron withdrawing or electron-donating groups did not exert any qualitative effect upon the enzymatic reaction. Under the reaction conditions, pH 8, the heterocyclic base of these substrates is assumed as completely or partially deprotonated. Therefore any participation of N_3-hydrogen atom of the pyrimidine moiety in the mechanism of the enzymic reaction, as postulated earlier[77], can hardly be expected. These findings are in agreement with those obtained in the case of compounds whose heterocyclic bases lack any hydrogen atom at the position corresponding to N_3 of uracil, namely the 2-pyridone derivatives[78] and the 2-pyrimidinone derivatives[79]. In previous work in this laboratory[69] it was shown that substitution of halogens at the 5-position of nucleotide cyclic phosphate does not have a marked effect on their hydrolysis by PRNase. Further work on the hydrolysis of 5-hydroxyuridine 2',3'-cyclic monophosphate showed similar results as reported in the appendix of this thesis. For these reasons efforts were diverted from the 2',3'-cyclic monophosphates to polymer synthesis in this project.

Biological Activity of Nucleotides

Some of the early work on the biological activity of nucleotides include those on oligodeoxynucleotides[149]; complexes of poly(A).poly(U) or of poly(C); and methylated bovine serum albumin[150] in which these polynucleotides were reported to stimulate formation of antibodies in mice. It was observed that

the double stranded poly(I).poly(C) and poly(A).poly(U) were active while the single stranded poly(I), poly(C), poly(A) and poly(U) were inactive[151]. Some naturally occurring polynucleotides like Helene extract from Penicillium funiculosum[80] and Statolon from Penicillium stroloniferum[81,82] were found to confer resistance to viral infection. The common feature in the biologically active polynucleotides is double strandedness[83]. Rabbits and mice were found to show production of interferon and resistance to viral infection when injected with poly(I).poly(C). Park and Baron[85] showed that poly(I).poly(C) treatment allowed rabbits to recover from Herpetic kerato conjunctivitis (an eye disease). Younger and Hallum[86] have shown that poly(I).poly(C) was similar to endotoxin rather than viruses in its mechanism of interferon stimulation. However, poly(I).poly(C) is toxic to cells[87] and even small doses of this complex can cause fever[88], and exert embryotoxic effects in rabbits and cerebellar symptoms and death within one hour in young chickens[89]. It was found that by complexing with DEAE-dextran the activity of poly(I).poly(C) is increased 100 fold in rabbit kidney cells[91], human leukocytes and human amniotic membrane cells[92] as well as chick embryo fibroblasts[93].

It has recently been shown that it is unlikely that complexing polynucleotides with DEAE-dextran causes increase in RNase resistance. It is more likely that this complexing with DEAE-dextran increases the uptake of the polynucleotides by cells[94].

Interferon

Interferon was discovered by Isaacs and Lindenman[98] as a substance which interfered with viral replication. It was found to be nondializable, sensitive to proteolytic digestion with trypsin, stable between pH 2 and 10 although isoelectric between pH 6.5 and

7.0, heat stable and insensitive to antibodies against the strain of influenza virus used to induce its formation. Interferon was found to be a glycoprotein[99-101]. The evidence was obtained from isoelectric focussing[102] in which the phenomenon of polymorphism as known in glycoproteins was observed[103]. Schonne et. al[104] in their experiments concluded that interferon is a glycoprotein possessing a variable content of sialic acid and exhibiting a peripheral heterogeneity (differences in the terminal saccharide residues[105]). This has been confirmed by Dorner et. al[106]. When RNA synthesis is inhibited either with virus[107] or actinomycin D[108,109], interferon production is reduced or totally eliminated. This has been observed in rabbit kidney cells[91-95], chick embryo[93], human leukocytes and amniotic membrane cells[96,97]. This perhaps indicates a de novo synthesis of interferon rather than production from a preformed pool. It may also be that interferon is synthesised from mRNA transcribed from a host gene rather than from viral genetic material. The interferon produced by one cell type is biologically active only in cells of that same species but against a variety of viruses[110,111] and sensitivity of animal viruses to a given preparation of interferon is known to exhibit a lack of virus specificity[112]. But interferons do not directly inactivate virus particles[113,114] nor do they inhibit virus adsorption, penetration or uncoating[115] and although synthesis of both viral RNA and protein is inhibited in cells pretreated with interferon, this inhibition is not seen if the host RNA and protein synthesis are eliminated during pretreatment[116]. It could be that interferon causes a stimulation of synthesis of an antiviral protein which then acts by inhibiting the translation of viral RNA[116-118]. Interferon can be induced by almost all classes of animal viruses in appropriate hosts[119,120]; polyanions like Penicillium stoloniferum[123] and Penicillium funiculosum[124,125];

pyran copolymers of maleic anhydride; polyacrylic and polymethacrylic acids[126], just like nucleic acids[127].

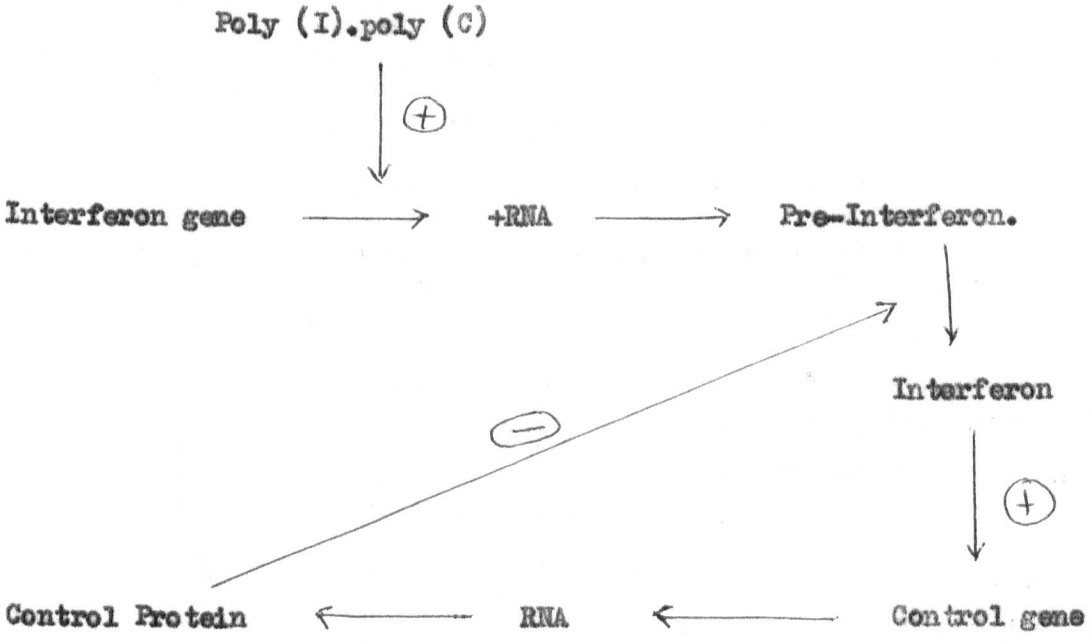
Interferon has been used for various treatments since methods of its purification have been developed[128]. By local application, Merigan has employed interferon to prevent virus infections of the respiratory tracts[129]. Merigan is also currently using interferon clinically against Herpes zoster but there are no clear-cut beneficial results yet. Interferon has also been tried as a potential suppresser of rejection in transplant patients[152].

Work has been done to investigate the specificity of interferon induction by various polynucleotides and it was shown that the resistant state of poly(I).poly(C) treated cultures is mediated by interferon[93,95,130-132]. Interferon induction by polynucleotides has no requirement for specific base sequences[80,83,133] and, synthetic double stranded polynucleotides having sequences A, U, G and X have been found to be active inducers[93,134,135]. There is however, a requirement for a stable secondary structure[91,93,134] and although some single stranded polynucleotides have been reported active, they are far less active than the double stranded helices[136,153]. Thus, there is a requirement for double strandedness and this will be discussed further later in this thesis.

Mechanism of Interferon Induction

Several questions can be asked as to how double stranded polynucleotides induce interferon. Two models are represented in schemes 7 and 8 but these do not tell exactly how those two strands behave before, during or after interferon production. The likely questions are put diagrammatically in scheme 9, raising at least five questions; do both strands stay outside the cell together? Does one strand get attached to the cell surface while the other strand either diffuses out or enters the cell? Does one strand remain on the outside of the cell while fragments of the other

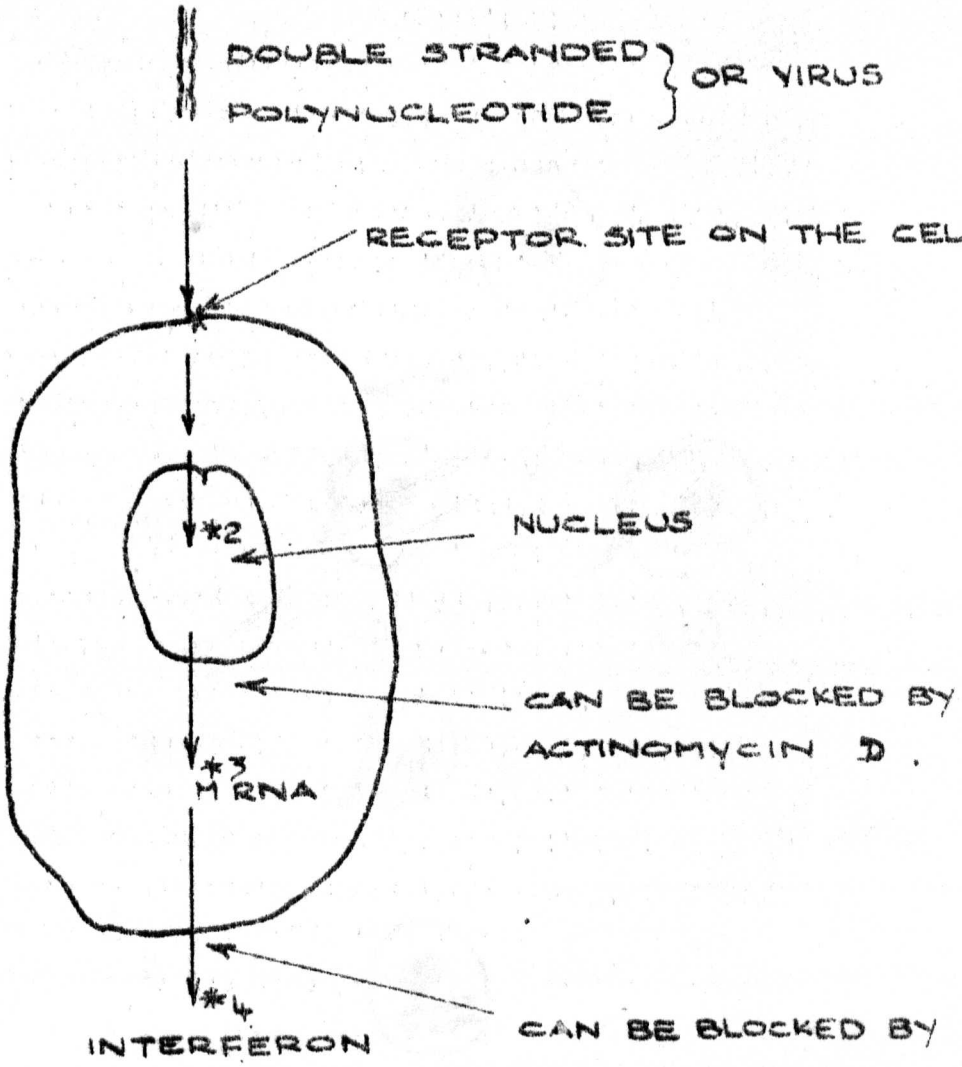
DIAGRAMMATIC REPRESENTATION OF INTERFERON INDUCTION BY Poly (I).poly (C).



Facilitation ..... +

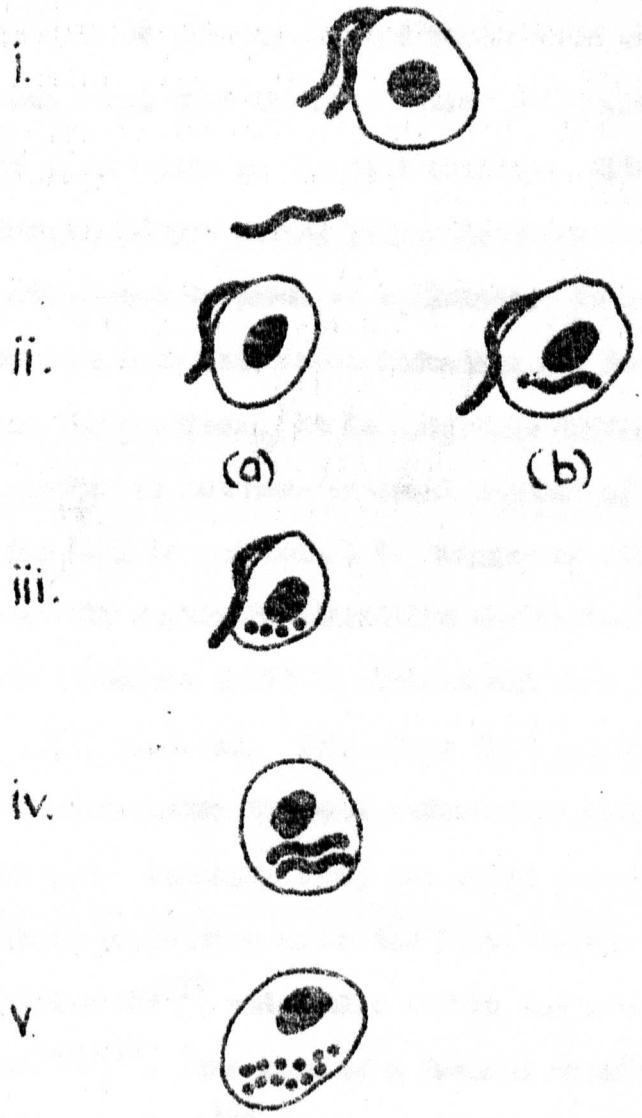
Inhibition ..... −

Scheme 7



POSSIBLE MECHANISM OF INTERACTION OF A DOUBLE STRANDED POLYNUCLEOTIDE WITH A CELL DURING INTERFERON INDUCTION.

SCHEME 8.



FURTHER ELUCIDATION ON THE POSSIBLE MECHANISM OF INTERACTION OF A DOUBLE STRANDED POLYNUCLEOTIDE WITH A CELL DURING INTERFERON INDUCTION.

SCHEME 9.

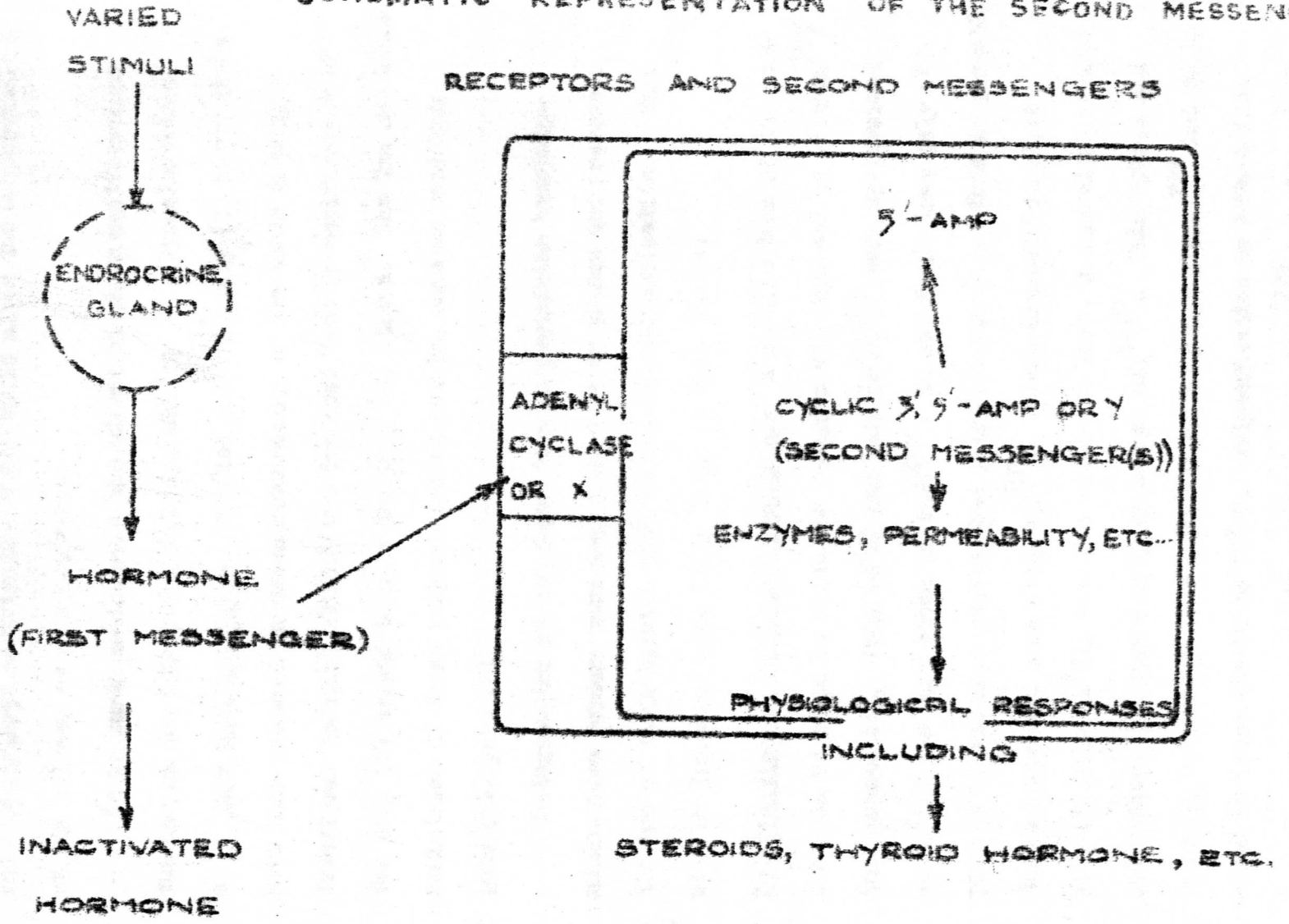
enter the cell? Do both strands enter the cell? Or, do fragments of both cells enter the cell? De Clercq et al.[137] studied a large variety of RNA and DNA bound or not bound to DEAE-dextran, their rate of binding to, and persistance on the cell surface. It was found that the more active polynucleotides exhibited some loss of activity on the cell surface. Pitha and Pitha (in press) thought of preventing polynucleotides from entering cells by attachment to powdered cellulose. It was also observed that there were both interferon induction and loss of polynucleotides from the carriers. It is therefore difficult to say categorically whether or not mere external contact of the polynucleotide on the cell is sufficient to trigger of interferon production. But recently Taylor-Papadimitriou and Kallos[138] have shown that loss from sepharose could be avoided and when this is done interferon is still produced. This shows that polynucleotides do not necessarily have to enter the cell before they induce the production of interferon. Perhaps, then, one could explain the mechanism of interferon production using the illustration of the mode of action of cyclic AMP[139] and cyclic GMP in the mitogen-induced clonal proliferation[140-146]. The idea of a "second messenger" formerly proposed by Sutherland[154] (see scheme 10) has been applied in the mechanism of interferon production[147]. But as Burke[155] has put it, there are a number of questions which only time can answer in the mechanism of interferon induction as to the precursor of interferon, where it is found in the cell, what changes occur in the activation and release of interferon and a number of other questions.

Synthetic modifications in polynucleotide

There are three main ways of modifying polynucleotides.
(a) Base modification which can either cause mismatching or which may cause the two strands of the helix to associate more strongly.
(b) Sugar modification:- This is alteration of the 2' hydroxyl leading to increased resistance to nucleases.

Scheme 10.

SCHEMATIC REPRESENTATION OF THE SECOND MESSENGER CONCEPT.



(c) Phosphoryl modification which would alter the susceptibility of the polymer towards nucleases.

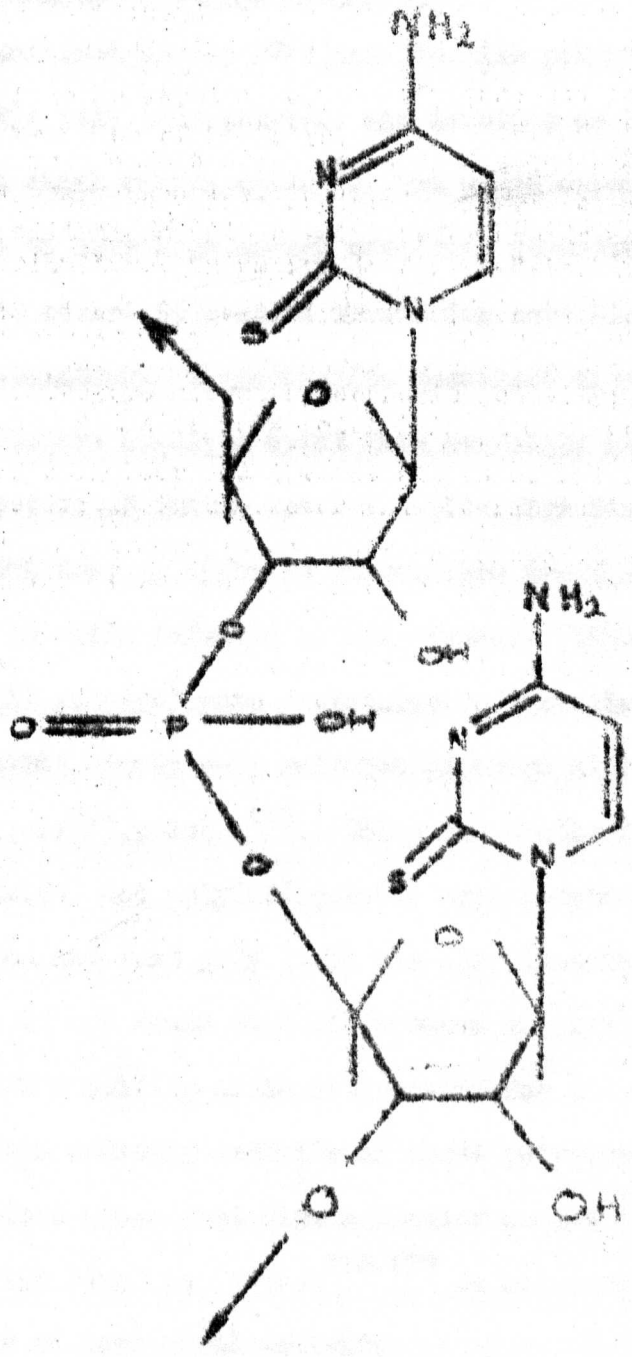
It is worth considering the effect of these modifications further on the interferon inducing ability of polynucleotides.

a) Base modification:- Modified poly (I).poly (C) derivatives have been prepared in which mismatching of the bases occurs. Interferon inducing ability was impaired less by modification of the poly (I) strand than by the poly (C) strand. The Tms and RNase resistance of these polymers are almost the same as unmodified poly (I). poly (C).

Halogenation at the 5-position in pyrimidine homopolynucleotides renders them more resistant to RNases and increases the Tms of their hybrids with purine homopolynucleotides. This will be discussed more fully later in this thesis.

Modification of the 2-keto position:- A modification of the base has been reported in which the 2-oxygen was replaced by sulphur[217] (see scheme 11). Poly (s^2C) and poly (s^2U)[218] were synthesised and s^2CMP has been shown in tRNA[219]. It was found that poly (I). poly (s^2C) was more stable than poly (I).poly (C). This is probably due to increased base stacking[220]. PRNase did not hydrolyse poly (I).poly (s^2C), but this enzyme is known to hydrolyse helical polynucleotides very slowly or not at all[9,18]. This hybrid did not melt between 10° C and 100° C and ethylene glycol[221] had to be added to lower the Tm to 77° C. Polymerisation by PNPase from E. coli does not normally exhibit a lag phase[220] but when s^2CDP was polymerised there was a marked lag phase which could only be abolished by the use of primers[18]. Poly (s^2C) shows a weak negative circular dichroic band at 325 nm of n-π^* transition[218] which is shifted to shorter wavelengths upon protonation or hydrogen bonding[223,224] just like in the CD spectrum of poly (A)[225].

Replacement of 2-keto by sulphur



Scheme 11.

It has been shown that vertical interactions mainly contribute to the stability of polynucleotides[223,226]. One could expect this phenomenon to be true of substitution into the 2-position.

Modification at the 2'-hydroxyl group:-

As mentioned above, DNAs and DNA-like polynucleotides e.g. poly (dA-dT); poly (dG).poly(dC) are inactive as interferon inducers in chick embryo cells[93]. One would expect these polynucleotides to have high enough secondary structure and should therefore be active if what is needed for activity is just secondary structure. These results therefore signify that there are other factors involved apart from secondary structure. Field et al.[83] postulated that a special replicative form of DNA or viral DNA-RNA complex might be responsible for the induction of interferon in cells infected by DNA viruses. Vilček et al.[91] found poly(I).poly(dC) gave resistance to vesicular stomatitis virus in rabbit kidney cell cultures although at 10^4 fold concentration as poly(I).poly(C)[134]. Colby and Chamberlin[93] found that poly(I).poly(dC) and poly(dI).poly(C) were inactive at 10 µg/ml concentration and that poly (A-U) was also inactive. From further experiments it was found that differences in permeability and intracellular stability could not account for the difference in the interferon inducing activity of these polynucleotides[134]. It is therefore clear that high molecular weight[175,177], high Tm[135] and high RNase resistance[178,179] do not account for all the requirements of biological activity.

A number of substitutions have been made into the 2'-position to investigate the importance of the 2'-hydroxyl group. Examples are poly (2'-OAsC)[180], poly (2'-cl C)[86], poly(I).poly(dC)[93,157], poly (I).poly (2'-OMe C), poly (A).poly (2'-OMe U)[127], poly (A).poly (2'-fl U)[181] and poly (A).poly (2'-cl U)[182]. Most of these

polymers have high Tm and are resistant to RNase[183,184]. Reports on bond angle studies by Voet and Rich[185], on NMR studies by Cushley et al.[186] and Suck et al.[182] showed that there was little difference in the molecular structure of these polymers and the unsubstituted polymers. For instance, there is no difference between the structure of poly (2'-cl U) and poly (U). However, this does not mean that changing the 2'-position does not alter the molecule in certain respects. For example, in the presence of magnessium ions, poly (U) and poly (2'-OMe U)[187] form helical structures while poly (dU)[188] and poly (2'-cl U) do not. Suck et al.[182] suggested that this difference is due to solvation rather than conformational effects, possibly with complexing of the metal ions to the 2'-oxygen. Fluorine in poly (2'-fl U) was expected to act as a hydrogen bond acceptor[189] and may be expected to effect the stability of single and double stranded conformations substantially provided that hydrogen bonding involving the 2'-hydroxyl group is a factor in stabilization. Similarly, the rates of hydrolysis of the phosphorodiester linkage by various nucleases will be affected. From the RNase and alkaline hydrolysis data obtained it was apparent that the substitution of the hydroxyl function by fluorine inhibited cleavage of the phosphorodiester bond. This may not be surprising since 2'-fluoro group cannot be expected to participate in the formation of a 2',3'-cyclic phosphate intermediate. Poly (2'-fl U) formed a 1 : 1 hybrid with both poly (A) and poly (X). Poly (A) forms 1 : 1 and 1 : 2 hybrids with poly (U), 1 : 1 with poly (d U) and none with poly (2'-amino U)[190,192]. Poly (X) forms only 1 : 1 complex with poly (U) and none with poly (d U)[193,194]. The Tm of poly (A).poly (2'-cl U) in 0.1M salt pH 7.5 is only higher by 2° C than the Tm of poly (A).poly (U)[195] which is lower by 11.5° C than the Tm of poly (A).poly (2'-OMe U)[187]. Poly (U).poly (X)[193,194]

appears more thermally stable than poly (2'-fl U).poly (X). In the pH titration of poly (2'-fl U) the increase in absorbance at pH 4 is said to be much lower than expected for the supercoiling reaction[181]. These variations observed with respect to different substituents in the 2'-position may have to do with the substituents themselves[196]. Again, 2'-amino UDP is a better substrate for polymerisation by PNPase than 2'-azido UDP[195,198]. From the CD data, a considerably lower degree of ordered structure for poly (2'-amino U) than poly (U) at 18°C was indicated but the structure of poly (2'-amino U) is in the anti conformation[195,199-203]. It has been shown that there is a wide spread distribution of 2'-OMe nucleotides in RNA from many organisms[204-209]. The 2'-OMe groups which are incorporated at the 45 S precursor stage are believed to be retained in the 28 S and 18 S products although about half of the nucleotides present at the start in 45 S RNA are eliminated in the maturation process[210,211]. And although adjacent pairs of 2'-OMethylated species occur in natural RNA[212], the tendency for cooperative incorporation apparently does not extend beyond two adjacent 2'-OMe nucleotides, Physical measurements on poly (2'-OMe A) showed that there is greater tendency for ordered structure than in poly (A)[213]. On the whole, the antiviral activities of these polynucleotides are lost as soon as the 2'-hydroxyl group is replaced.

In a recent work in this laboratory[214], the problem of the 2'-hydroxyl group was studied. Poly (cl^5 C), poly (br^5 C) and poly (i^5 C) were hybridised with poly (d I). These hybrids were found not to induce interferon in human embryo fibroblast cells as shown in the table below.

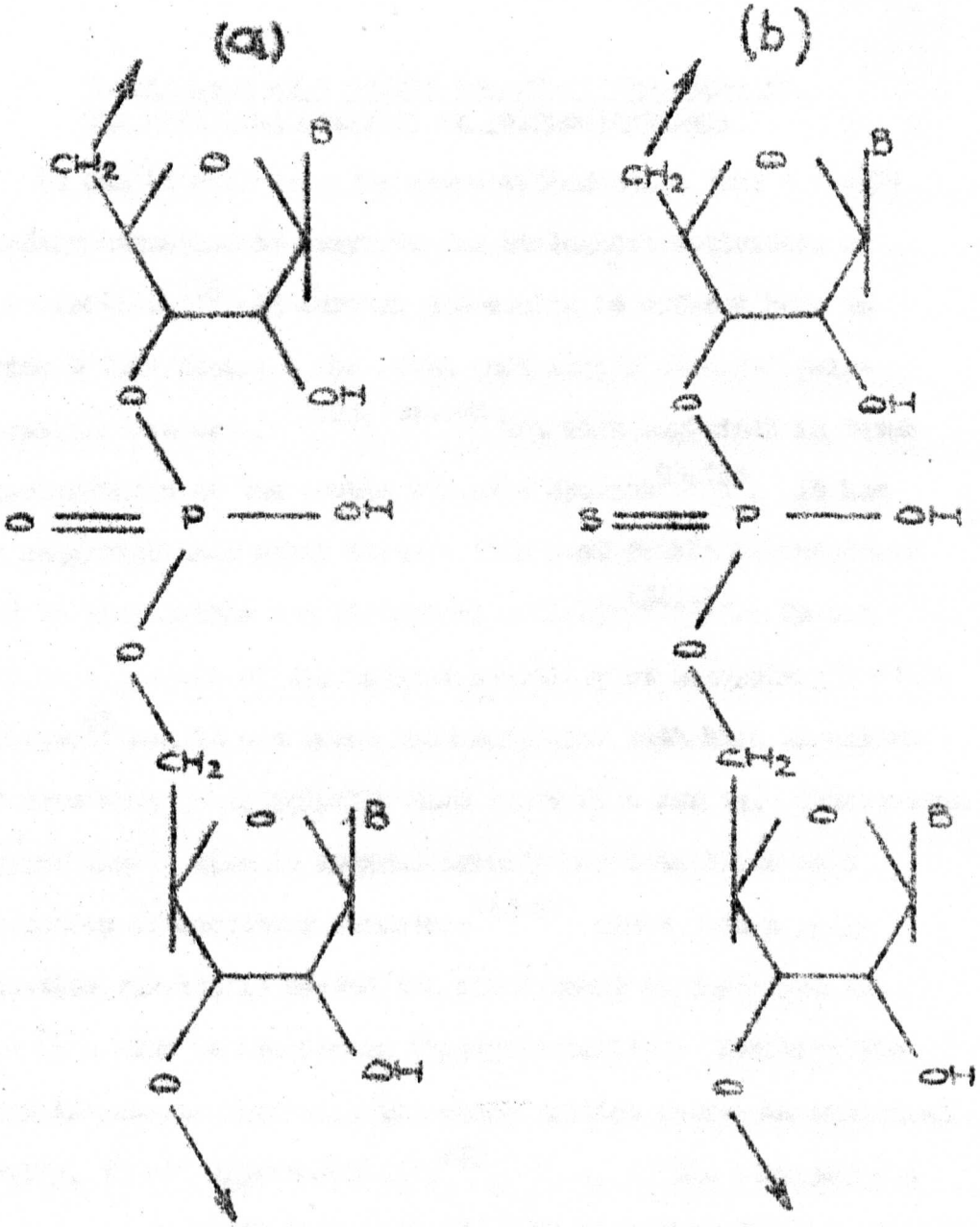
Effect of 2'-OH on Interferon yield[214].

Polynucleotide Hybrid.	Tm °C 0.1 M Na^+	Interferon Yield. (Units.)
Poly (dI).poly (C)	35	2
Poly (dI).poly (el^5C)	57	2
Poly (dI).poly (hr^5C)	66	2
Poly (dI).poly (i^5C)	68	2
Poly (I).poly (C)	61	55

De Clercq et al.[215] tried a variety of 2'-modified polynucleotides and found their antiviral activities on primary rabbit kidney cell cultures. The results obtained showed that the presence of a 2'-hydroxyl group is a requirement for interferon induction.

Modification of the phosphate backbone:- Another position that has been modified is one of the oxygens of the phosphate group not involved in the phosphodiester linkage. This has been replaced by sulphur[216]. This polymer hybridises with poly (U) and the hybrid has a Tm of 48° C in 0.01M sodium chloride solution, which is the same as unsubstituted poly (A) hybridised with poly (U). The thiosubstituted polymer has a higher RNase resistance and higher interferon inducing ability. One could argue that because the Tm is the same as that of poly (A) the stability of the helix has not changed by the replacement of an oxygen by sulphur and that increased RNase resistance is due

Replacement of phosphate oxygen by sulphur[216].



Scheme 12

to an inhibitory action of sulphur atom at the active site of RNase. Also poly ($A^{S}U$) may have a higher affinity than poly (A-U) for the intracellular site that triggers the production of interferon.

Requirement of a stable secondary structure for the antiviral activity of polynucleotides.

It can be seen from the above introduction that a stable secondary structure is required for biological activities of polynucleotides[135] and further discussion is offered here to elucidate this theory. The claim that single stranded polynucleotides are active[91,95,164-168] has been explained in terms of contaminants of the double stranded species[95,131]. It has been suggested that other factors than just double strandedness could be responsible for biological activity[134,135]. Tm was taken as a measure of the helical stability of homopolynucleotide complexes[63] and it was found that complexes with high Tm values were more active biologically than those with low Tm. Observation of hyperchromic rise in optical density has been taken as a destruction of secondary structure[169-171] since when a polynucleotide complex is heated the arrangement is destroyed and there is a rise in absorbance (hyperchromicity). Lowering the pH and increasing magnesium ion concentration increases antiviral activity, Tm and hyperchromicity[172]. Thus, double strandedness is necessary for antiviral activity. It will therefore be of interest to be able to increase the ability of polymers to form stable double strands. It has been shown that antiviral activity was related to Tm between 40° C and 60° C[135]. Above 60° C it is not directly related to Tm, but since high Tm is an indication of stable secondary structure[173,174] it will be worthwhile to explore the possibility of enhancing this.

The above argument is supported by Colby and Chamberlin[93] who treated chick embryo cells with various synthetic polynucleotides

and challenged them with Sindbis virus and some polynucleotides produced interferon while some did not. Their results are shown below.

Inducing	Non-inducing
Poly(I).poly(C)	Poly(I)
Poly(I-C)	Poly (C)
Poly(I-br^{5}C)	Poly (G)
Poly (G).poly(C)	Poly (A)
Poly(A).poly(U)	
Poly(A-U)	Poly(A).Poly(U)$_2$
Poly(A-br^{5}U)	Poly(dI)$_2$.Poly(C)
	Poly(I).Poly(dC)
	Poly(dI).Poly(dC)
	Poly(dG).Poly(dC)
	Poly(dA-dI)
	Poly(A-dU).

The single strands of poly(I), poly(C), poly(G), poly(A) and poly(U) were inactive at 10 µg/ml. These also agreed with other results[153].

DISCUSSION

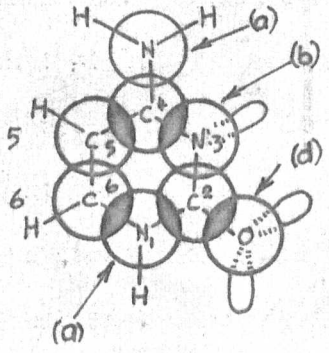
Modification at the 5-position of pyrimidine and 8-position of purine bases.

This topic will be considered in more details here as it is the basis for this thesis.

Purine and pyrimidine bases of nucleic acids are cyclic systems composed of interlinked trigonal (SP^2 hybrid) atoms. The p-electron atomic orbitals intersect to form π-electron molecular orbitals. A pseudo aromatic system thereby arises (see schemes 13-15). The mode of electron density distribution shows that the electron density in the pyrimidine base is higher at C-5 than at C-6. It can be seen that the C-8 atom of purine bases will possess some deficiency of electron[227] like the C-5 of pyrimidine bases. One might therefore expect attack by an electrophilic agent to occur at the C-5 in pyrimidine and at the C-8 of the purine although less readily in the purine base than in the pyrimidine base. This is why, for instance, the pyrimidine base is brominated to completion in 30 minutes whereas one needs several hours to brominate the purine base. For this reason this project has been designed to investigate the possibility of substituting into the 5-position of cytidine and 8-position of adenosine. This has been encouraged by earlier work by other authors as summarised below.

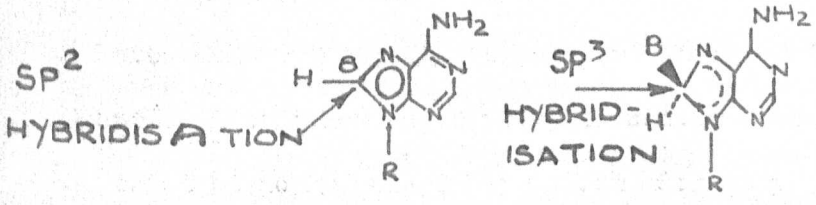
Poly (5-chloro[230]-, 5-bromo[228]- and 5-iodocytidylic[220] acids) have been prepared by the polymerisation of the corresponding nucleotide diphosphates using PNPase. These polymers were all more resistant to hydrolysis by PRNase than poly (C). Their hybrids with poly (I) were active inducers of interferon and had melting temperatures of 75°C, 83°C and 91°C respectively in 0.1M salt[93]. These temperatures are all higher than the melting temperature of poly (I).poly (C) hybrid which is 61°C[81]. Similarly poly (5-hydroxycytidylic acid) was prepared by the polymerisation of the diphosphate[231]. Poly (ho^5C) did not form an appreciable hybrid with

Scheme 13.
Aromaticity in Pyrimidine and Purine bases.



(a)

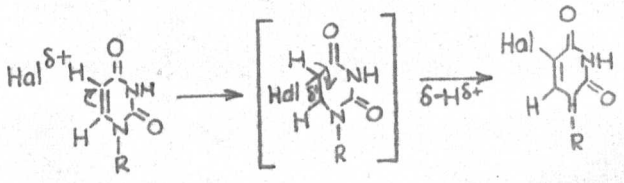
THE CYTOSINE MOLECULE AS AN EXAMPLE OF A CONJUGATED SYSTEM WITH HETEROATOMS OF TYPES a, b, and d.

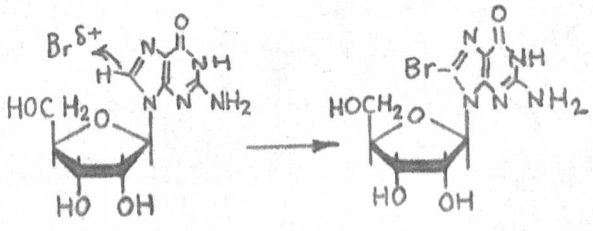


(b.)

CONJUGATION IN THE ADENOSINE BASE RESIDUE

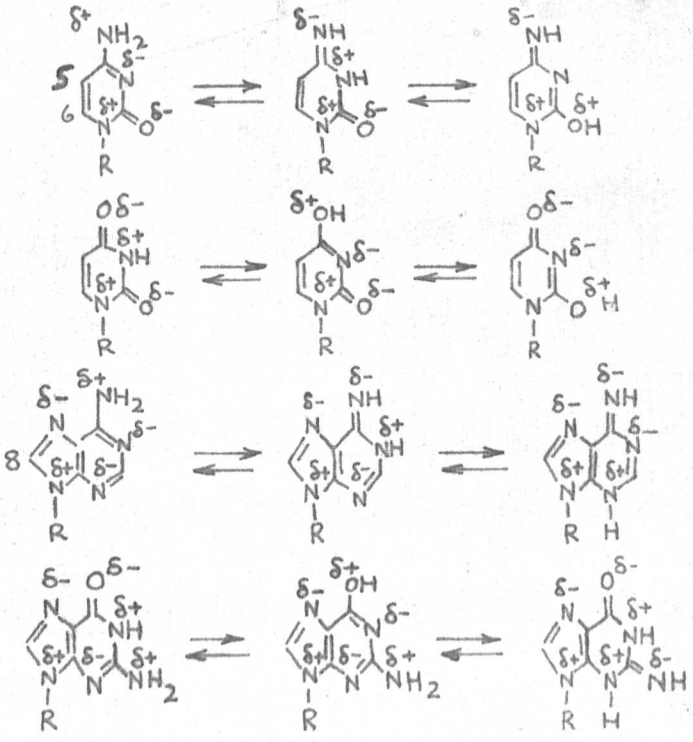
Electrophilic Substitution in pyrimidine and purine bases.





Scheme 14 .

Electron conjugation in some base residues of nucleotides



Scheme 15

poly (I) and an equimolar mixture of poly (ho^5C) and poly (I) failed to induce the production of interferon but causes resistance of cells to viral infection.

Poly (5-fluorocytidylic acid)

All the 5-halosubstituted polycytidylic acids mentioned above exhibit very high melting temperatures and although they formed 1 : 1 hybrids with poly(I), with these hybrids inducing the production of interferon, they did not induce more than the hybrids of unsubstituted poly(C) with poly(I). To complete the series of the halogens, it was suggested that poly ($f1^5C$) be made, and poly ($f1^5C$) is of particular interest because it was reported earlier that 5-fluorocytidine is found in RNA from *E. coli* grown in the presence of 5-fluorouridine[232].

A great deal of problems were encountered in finding a suitable fluorinating agent. From this table it can be seen that it will be very difficult to obtain a situation such as:

$$F_2 \longrightarrow F^+ + F^-$$

	F	Cl	Br	I
Electron structure	$S^2 2S^2 2P^5$	Ne $3S^2 3P^5$	Ar $3d^{10} 4S^2 4P^5$	Kr $4d^{10} 5S^2 5P^5$
Oxidation States	-1	±1,4,5,6,7	±1,4,5.	±1,4,5,7.
First Ionisation Energy K cal/g.Mole	402	300	273	241

Fluorine has only one oxidation state and this is negative. This is the reason for the problems encountered in this current work to fluorinate cytidine. Earlier work in this laboratory[230] showed some difficulty in chlorinating cytidine too. Chlorine being a gas could not be employed in the same way as bromine or iodine and a weak salt of tetrabutylammonium iodotetrachloride had to be

employed to generate chlorine which in turn did the chlorination.

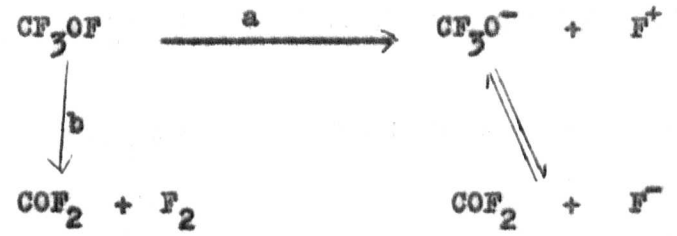
$$(t\ But)_4\ N\ I\ Cl_4 \rightleftharpoons I\ Cl_2^- + Cl_2 + (t\ But)_4N^+$$

But a fluorinating agent was not as easy to obtain as this chlorinating agent.

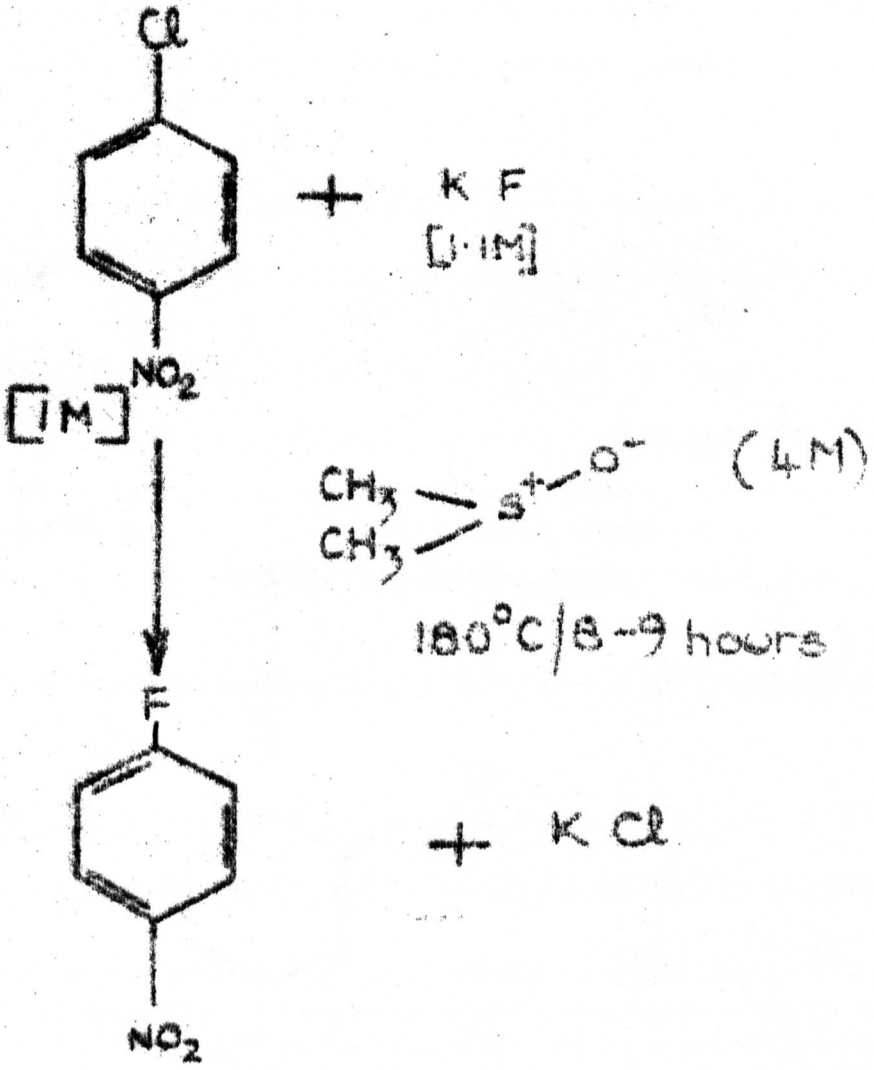
It has been found that an aromatic chlorine or bromine atom activated by o- or p- nitro group was subjected to nucleophilic displacements by fluoride ion on reaction with potassium fluoride in succinonitrile, DMF, or DMSO[74] (scheme 16). Other agents used include AgF_2, KHF_2, KF, HF, ZnF_2. When all these were tried on 5-bromocytidine in the present work there was no replacement of bromine by fluorine even though in some respects the reactions of a pyrimidine ring resemble those of dinitrobenzene.

Trifluoromethyl hypofluorite (CF_3OF) has been employed to fluorinate several delicate compounds. Keto-, alkoxy-, acyloxy-groups have been reported to survive this reaction[228]. OH and NH groups have been found to be acylated to some extent by the traces of carbonyl difluoride present. Acylation could be prevented by adding methanol to the reaction mixture. Alternatively the acyl groups could be removed by hydrolysis with aqueous sodium carbonate during the work up. Further use has been made of trifluoromethyl hypofluorite to fluorinate the benzene ring[233] (scheme 17) but has been found not to react with methane, chloroform or carbon tetrachloride at room temperature. Another fluorinating agent, perchloryl fluoride ($FClO_3$) has been employed,[234] but this resulted in mixed fluorides and chlorides as products. This reagent does not react with unactivated olefines.

Mechanism of CF_3OF Reaction[235,236]

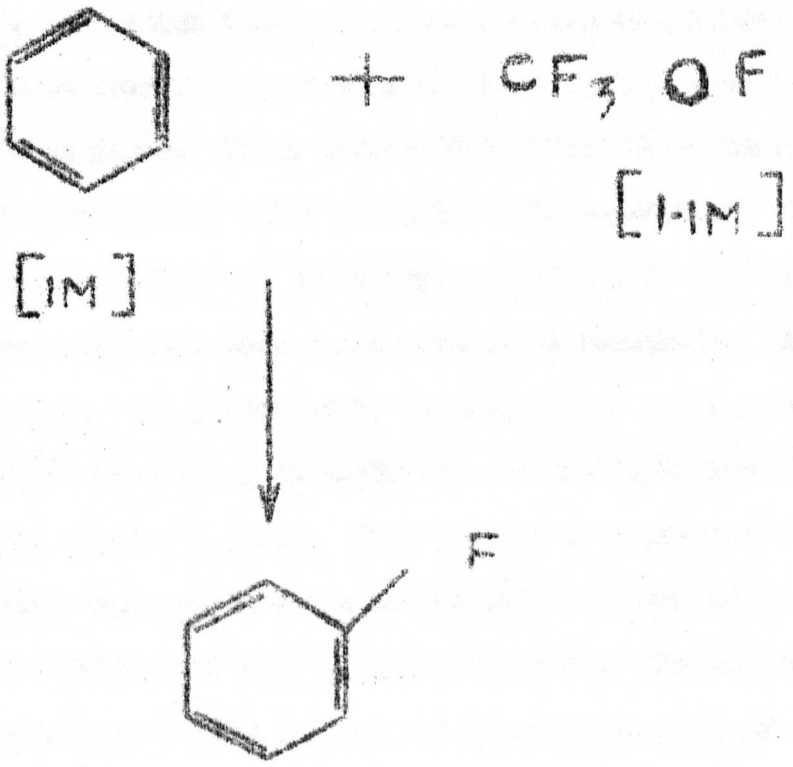


Replacement of Chlorine by Flourine



Scheme 16

Direct Flourination of benzene by trifluoromethyl hypoflorite.

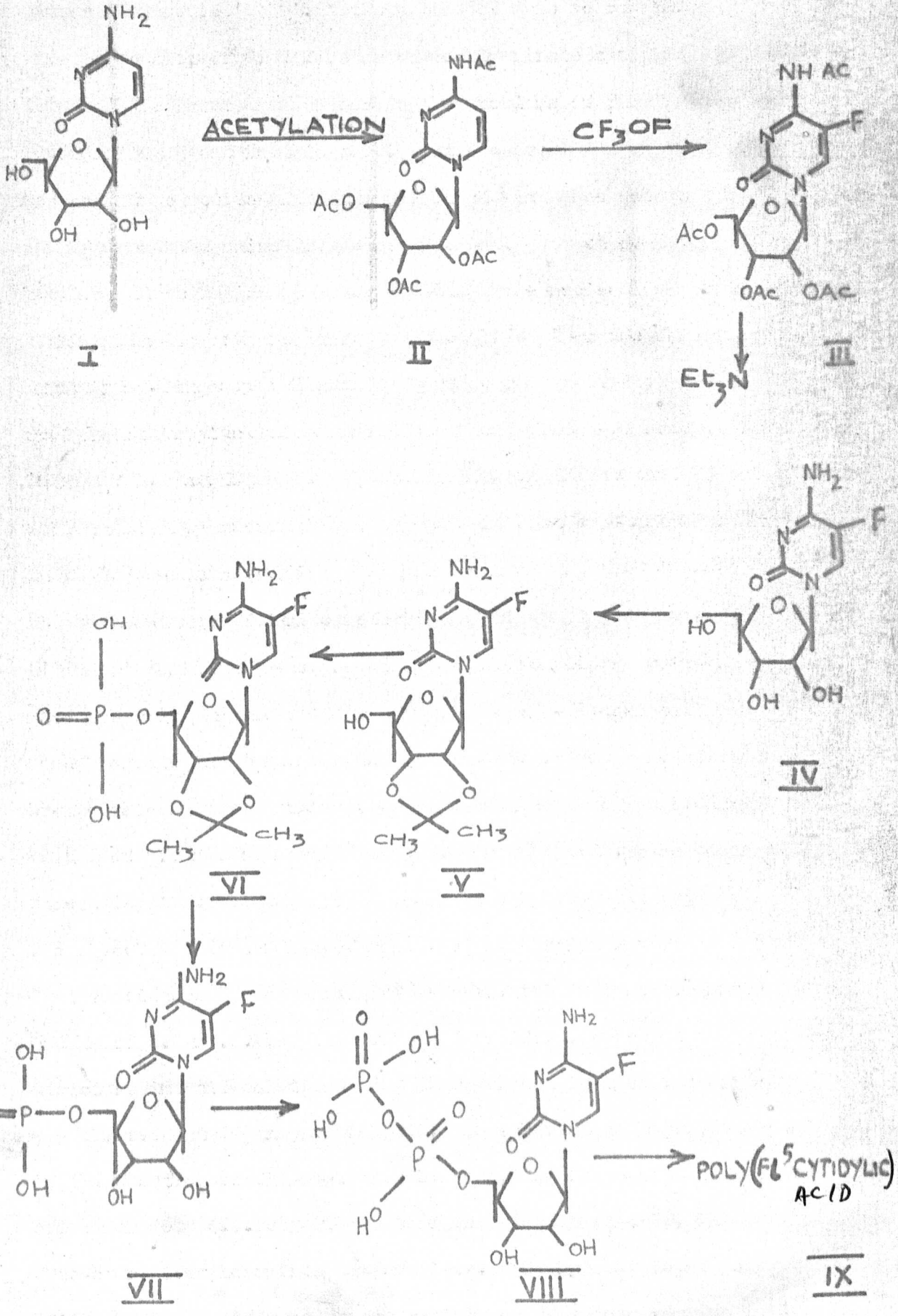


Scheme 17.

Two possible mechanisms have been postulated 'a', and 'b', but it has been shown that reaction proceeded through route 'a' rather than route 'b'[237]. At the time of this search for a fluorinating agent, CF_3OF was reported to fluorinate cytidine[161] and this method produced an answer to the problems of finding a suitable fluorinating agent in this work. But one great danger is that this reagent is so active that it could react with phosphates, the hydroxyl and the amino groups of cytidine. To get round this problem, fluorination was carried out at the nucleoside level (see scheme 18) and the amino and the hydroxyl groups were protected by acetylation; a reaction which gave near quantitative yield. The acetyl groups were easily removed to yield free 5-fluorocytidine in about 70% total yield. Tests carried out showed that fluorination did not occur at any other place than the 5-position. The resulting 5-fluorocytidine was phosphorylated at the 5'-position by the phosphoryl chloride reaction in trimethyl phosphate. The 2' and 3' positions were protected by isopropylidine group which was also easily removed later to yield free 5-fluorocytidine 5'-monophosphate in about 40% yield. This product is converted to the corresponding diphosphate which was in turn polymerised by PNPase to give a polymer of $S_{20,w}$ 6.6 in 40% yield. The polymer made a 1 : 1 hybrid with poly (I) and the hybrid with a Tm of 66°C was a good inducer of interferon. Polymerisation was followed by the release of inorganic phosphate to give Vmax of 1.0 μMole and Km of 8.8 mM. The polymer had a considerable secondary structure in acid solution and a sharp change in the UV absorption was observed at 290 nm around pH 2.6 in contrast to a gradual change in the spectrum of $f1^5$ CDP. Furthermore, the shape of the melting curve for poly (I).poly ($f1^5C$) changes abruptly at 66°C, indicating a co-operative phenomenon taking place. The hydrolysis of poly ($f1^5C$) by PNase indicated more resistance than poly (C) but less than poly (br^5C) and poly (Cl^5C) respectively to PNase digest.

Scheme 18.

SYNTHESIS OF POLY (Fl^5C).



The CD spectrum of poly (fl^5C) resembles that of poly (C) and the other 5-substituted poly (C) indicating a common anti conformation.

Over the whole range of halo-subsituents into the 5-position of poly (C) there is a remarkable linear relationship between the melting temperatures (Tm) of the corresponding hybrids with poly (I) and the ionic polarisabilities of the substituents in poly (C). It is reasonable to look more closely at this polarisability effect. Polarisability of the molecule has been defined as the moment which is induced when an electric field of unit strength acts upon a molecule[80] and will be labelled 'b' in this discussion. Polarisability in a system like X - Y could occur in three ways.

It could be longitudinal b_l^{x-y},

It could be vertical b_v^{x-y} and

it could be transverse b_t^{x-y}.

To add to the complication, polarisability is also influenced by the other parts of the molecule. But on the whole, in a specific molecule with different atoms substituted, the properties like viscosity coefficients, Van der Waal's interaction, polarisability, molecular magnetic rotations, critical volumes, molecular sound velocities and molecular refractions can all be linearly interrelated. When the Tm's of poly (I).poly (X^5C) were plotted against

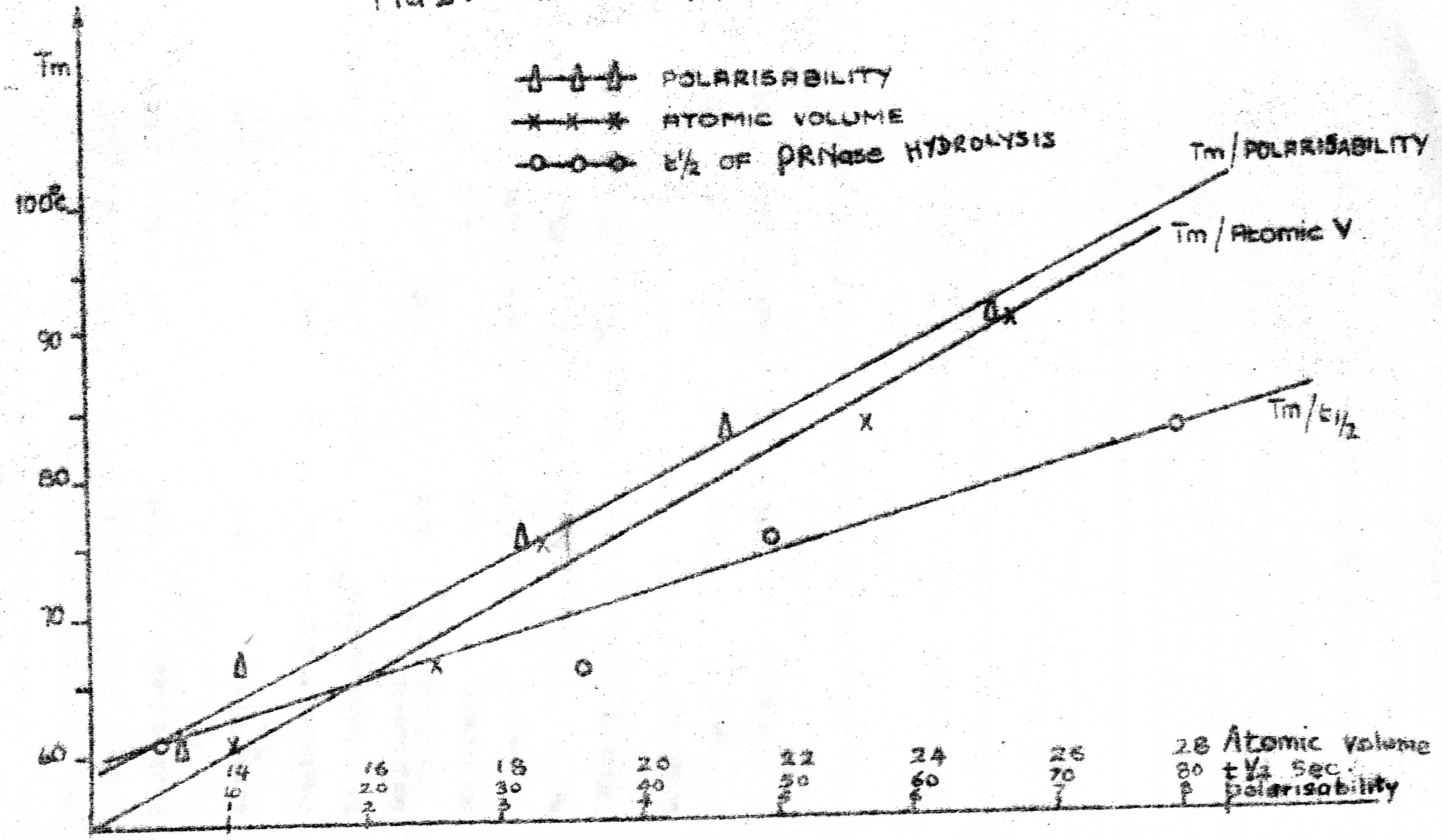
(1) Polarisabilities of X^{80} (2) Atomic volumes of X

(3) Ionic polarisabilities of X^{238} (4) $t_{\frac{1}{2}}$ of PRNase hydrolysis of poly (X^5C)

straight lines were obtained for X = H, F, Cl, Br and I (Fig. 2).

Perhaps one could endeavour to explain these relationships in terms of hydrophobic forces. These are the forces that arise when a molecule with a charge, a dipole or a group which can act as a donor or acceptor of protons or electrons binds to another small molecule or a protein in aqueous solutions[239]. This applies in the case of nucleic acids which despite their large dipole moments

Fig 2 :- Tm of POLY (I). POLY (X^5C) X = F, Cl, Br, I



X =	H	F	Cl	Br	I
Covalent Radius. A^{o}	0.37	0.72	0.99	1.14	1.33
Atomic Volume W/D	14.1	17.1	18.7	23.5	25.7
Electronegativity	2.1	4.0	3.0	2.8	2.5
Ionic Polarisability[80]		0.89	2.42	4.13	6.42
Longitudinal Polarisability[80]	0.65	1.2	3.18	4.65	6.70
pKa Polymer	5.82	2.45		4.43	5.00
'' Monomer	4.33	2.26	2.49	2.61	3.01
Tm ^{o}C	61	66	75	83	91 [220]
PRNase $t_{\frac{1}{2}}$ sec.	5	36	50	80	-
UV max nm.					
pH 7	268	281	287	289	298
pH 2	276	286	298	292	308

are highly hydrophobic in the sense that their solubility in water is generally small (i.e. they tend to act more strongly between themselves rather than with water). Addition of compounds such as alcohols, has been found to increase the solubility of hydrophobic compounds in water and similarly to increase the solubility of nucleic acids. It is not surprising therefore that it has been observed that lower Tms occurred for poly (I). poly (C) and poly (I).poly (br^5 C) when ethylene glycol was added and that the drop of Tm is proportional to the amount of ethylene glycol present[228]. Indeed Howard et al[240] have studied the effect of the substituent at the 5-position by looking at poly (I). poly (br^5 C) hybrid formation. It was found that Tms were linearly related to the amount of the 5-bromocytidine residues present in the hybrid formation and that the stabilising effect of bromine residues does not depend significantly upon sequence or Br-Br- proximity in the chain. The effect of ethylene glycol upon Tm is similar in poly (I).poly (C) and poly (I).poly (br^5 C) suggesting that differential solvation of poly (C) and poly (br^5 C) is probably not due to solvent effects or to in-plane I-C interactions within the helix but that may instead arise from the rather high polarisability of bromine acting upon cytidine residues in the same chain. Howard, et al[240] state also that the effect of introducing the bromine into position 5 alters the dipole of the cytidine residue and hence increases the in-plane attractive forces (i.e. dipole-dipole forces). These dipole-dipole forces appear to be even more important than more base stacking. The CD data confirms a trend for the polymers of 5-halo cytidine. They are all alike and this indicates that they are all in the same anti-conformation and strongly dichroic. It is even more interesting that similar linear relation has been observed for 5-halo substituted polyuridines by Massoulié et al[241]. The Tms of poly (A).poly (hal^5 U) were linearly related to the amount of the hal^5 U present and titration indicated stabilisation by

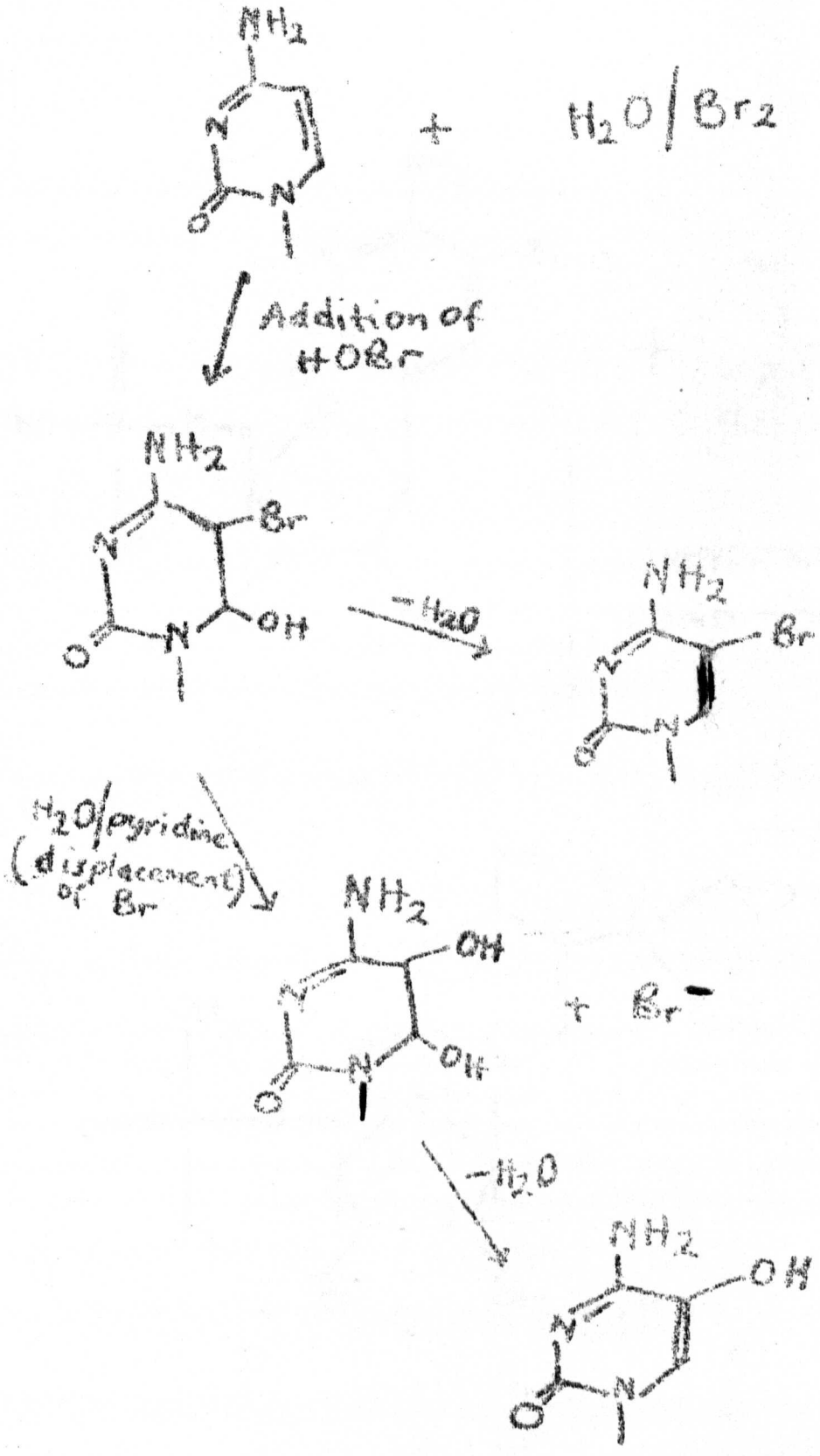
5-substitution on secondary structure. The stability of the secondary structure of the hybrid of poly (A).poly (hal^5 U) was also found to be related to the polarisability of the halogen substituent. It is on the whole, very interesting that poly (fl^5 C) fits into the regular pattern of the other poly (hal^5 C).

Poly (5-dimethylaminocytidylic acid).

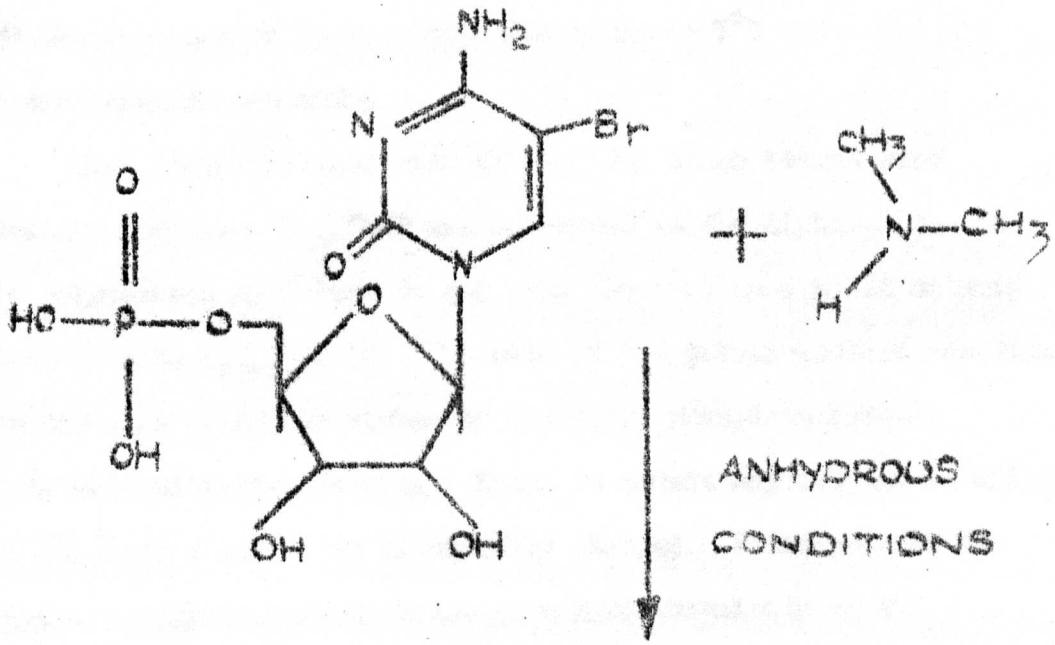
It appeared that all the 5-halosubstituted poly (C) followed a particular pattern with respect to their resistance to RNase and their Tms. This was also suggestive of a particular trend of secondary structure. Perhaps all these data fit in nicely because the substituents all belong to the same group and are all electron withdrawing. The next attempt was to substitute a group other than the halogens into the 5-position. Again, this met with some difficulties as several attempts made to insert various electron donating groups failed. But, 5-methylamino 2'-deoxy uridine has been known to enhance cell growth and cell division in a thymineless strain of E. coli[242] and to produce a potent, highly specific suppression of Herpes simplex virus propagation[243,244]. The synthesis of 5-methylamino and 5-dimethylamino uridine has been described[245]. These methods were employed, with considerable modifications, to synthesize Me_2N^5CMP. In this reaction a bromide ion was replaced by a dimethylamino group. The mechanism is not very certain but could be likened to the formation of ho^5 CMP. In fact if br^5CMP was first isolated, it could not be converted to the ho^5CMP in good yield. However, traces of the ho^5CMP were found in the synthesis of Me_2N^5CMP if the reaction mixture was not completely anhydrous. To get over this, DMF had to be used as solvent and the nucleotide had to be converted to the tetrabutyl ammonium salt to be soluble in dry DMF. Also, heating in sealed

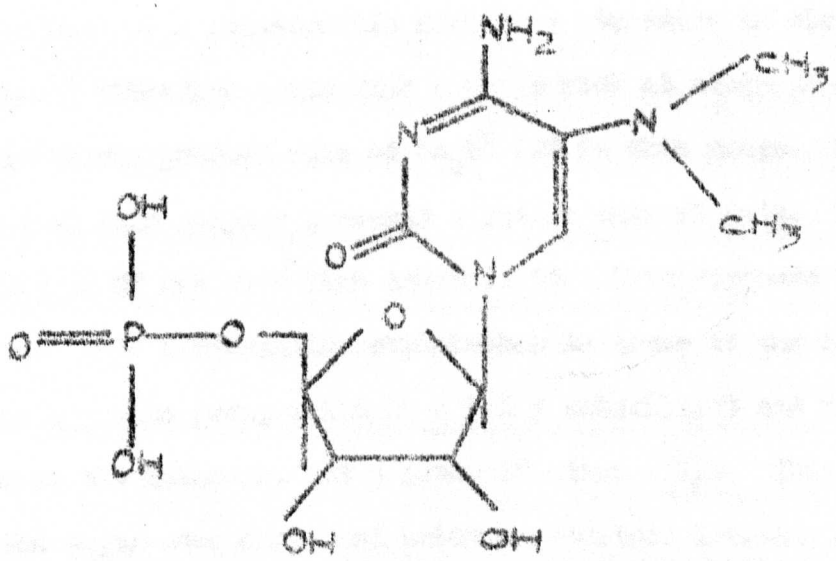
SCHEME 19.

Formation of 5-hydroxycytidine.[214]



Scheme 20.
Replacement of bromine in cytidine by a dimethylamino group





tubes or at very high temperatures led to deamination of cytidine, so the temperature had to be kept at about 60°C. This probably explained why the reaction never went to completion. There was always unreacted br^{5}CMP left and the yield of Me_2N^5 CMP was always about 32%, but always found pure by various tests. The reflux condenser had to be specially made to enable cooling by dry ice and acetone as dimethylamine boils at 7°C and would not be condensed by tap water.

Once these problems were solved, the other stages were straight forward. Me_2N^5CMP was converted to the diphosphate and was polymerised by PNPase to get poly (Me_2N^5C) in a yield of only about 7% with $S_{20,w}$ of 10. The rate of the polymerisation reaction was too slow to follow either by inorganic phosphate release or by radioactivity counting. Even, to obtain any polymer at all the conditions had to be drastically changed. Hydrolysis of the polymer by alkaline or by RNase gave hyperchromicity of 4% compared to 30% of poly (C) under the same condition. The rate of hydrolysis by RNase is slower than that of poly (C) ten fold. There is however a considerable secondary structure as observed from the pH titration curve with a sharp rise at about pH 4.1 compared to the gradual rise of Me_2N^5 CDP in this range. The CD spectrum of this polymer presents a rather unusual data. It has low positive CD and very high negative CD. This suggests that it is in the syn conformation explainable in terms of the size of the dimethylamino group which is a bulky substituent and not just an atom as the halogens, but a group of atoms C_2H_6N. This may alter the sugar-base angle and being an electron donating group will be expected to behave differently from the halogens on the pyrimidine base. Perhaps one can explain the low yields of polymerisation reactions in terms of this difference in conformation. The conditions for polymerisation have so far been those

under which the anti conformers are polymerised. It is interesting, however, that despite the seemingly different configuration from the other polymers of cytidine, it forms a 1 : 1 hybrid with poly(I) with a Tm of 58.5°C, a little lower than that of poly(I).poly(C). The hybrid was also found to be a very good inducer of interferon. Perhaps interferon induction has little or nothing to do with the conformation of the polymer. It was found that the Tm did not fall in the expected position in the plot of the polgrisability of 5-substituent against Tms of the hybrids of the 5-substituted polymers of cytidine with poly(I). It is of course not easy to work out the polgrisability of the dimethylamino group.

Poly (8-oxyadenylic acid)

Ikehara[246] reported some difficulties in obtaining homopolymers of 8-substituted purines from the corresponding diphosphate nucleosides. Copolymers were obtained with unsubstituted purines and the rate of this polymerisation was retarded in a trend proportional to the amount of 8-substituted species present. The explanation given was that either there was distortion, by large substituents sited at the 8-position, of the enzyme's active site(s) or there was a different conformation of the analogue diphosphate from the unsubstituted diphosphates. A crystallographic study by Tavale and Sobell[247] showed 8-bromoguanosine to have a syn conformation.

It was thought that the presence of syn conformation was due to very big groups in the 8-position of purines. It was therefore suggested that a smaller atom like oxygen be inserted. Ikehara[246] has reported that O^8ADP and O^8GDP would not be polymerised. But it was suggested that this polymerisation be attempted again

varying the conditions of polymerisation as in the preparation of poly (Me_2N^5C). And so, the oxy group was introduced into the 8-position of 5'-AMP by the replacement of a bromide ion in br^5AMP. The resulting O^8AMP was converted into the diphosphate which was then polymerised by PNPase. One important step in the replacement of bromide ion is the first formation of OAc^8AMP. This intermediate had to be refluxed in alkaline medium for 18 hours to convert it to free oxy group. This step was not recorded in the preparation of O^8AMP by Ikehara[246]. Perhaps this explains the problem encountered in getting the homopolymer from the said O^8ADP. However, the polymer obtained in this project had $S_{20,w}$ 7.2 and formed only 1 : 2 hybrid with poly (U), which had a Tm of 62°C. There was some difficulty in assaying the hybridisation between poly (O^8A) and poly (U). At equimolar concentrations the UV spectra of poly (O^8A) and poly (U) do not make any isosbestic point as poly (O^8A) has higher molar extinction coefficient than poly (U) between λ 200 and 400 nm. But when 1:2 concentration solutions of poly (O^8A) to poly (U) were made, it was easy to make the UV spectra intersect. So, by the method of continuous variations[259] the formation of a poly (O^8A).poly $(U)_2$ was demonstrated but no evidence could be found of a 1:1 hybrid. The homopolymer was less resistant to RNase hydrolysis than poly (A) probably due to the presence of syn rather than anti conformation. Since the polymer did not form the required 1:1 double stranded hybrid with poly (U) for interferon induction, no interferon assays were carried out on this polymer.

The polymer however, presented a big problem of lack of secondary structure. Sodium hydroxide and PRNase digest of it gave only about 1% hyperchromicity at λ 281 nm. Under similar

conditions poly (A) gave 36% hyperchromicity. By pH titration poly (O^8A) did not exhibit any sharp fall as for poly (A) but a gradual drop like O^8ADP in the region of pH 5 - 6. There was a fairly sharp drop in the region of pH 9 as compared to a gradual slope of O^8ADP in this region. The CD spectra of poly (O^8A) gave some problem too. There was a very low CD absorbance. It may be that the polymer was in a random disordered coil. The disruption of the assymetric helical conformation will result in the disappearance of the cotton effect and the resulting spectrum of the unstructured chain will be that of the sum of the monomer constituents, which will be weak[249]. Alternatively although most of the mononucleotides have been found to be in the anti conformation[250], poly (8-amino A) was said to be in the syn conformation just like some 8-bromo purines[84]. Perhaps poly (O^8A) is in the syn conformation too. The susceptibility of poly (O^8A) to PRNase suggests that poly (O^8A) is in the syn form as polyformycin which is in the syn form and is hydrolysed by PRNase. But whatever conformation poly (O^8A) is in, it is almost certainly in a random coil.

Conclusion.

Poly(5-fluorocytidylic acid), poly(5-dimethyl aminocytidylic acid) and poly(8-oxyadenylic acid) have been successfully synthesized. The first two polymers when hybridised with poly (I) have been shown to have considerable secondary structure while the third does not show this. The first two are also more resistant to PRNase digest than the corresponding unsubstituted polymer and the third one is not. The first two polymers have been found to be very good inducers of interferon but the third one could not be used as it did not form the required double stranded hybrid with poly (U). All these observations point to the fact that a

a stable secondary structure and resistance to RNase are important features in the mechanism of interferon induction.

On the whole the results of this project have been very encouraging in that they afford the possibility of overall discussion of a whole series of the halogens as substituents in polycytidylic acid. The difference observed by putting in a different group from the halogens (dimethylamino) is very significant. The difficulties encountered in the studies of poly(O^8A) indicate the presence of a random coil and probably syn configuration.

EXPERIMENTAL

Common Procedures

Chromatographic systems

A. Thin layer; silica gel developed with methanol: ethylacetate 50 : 50, v/v.

B. Paper; downward development with water (saturated with ammonium sulphate): sodium acetate: isopropanol (80 ml: 180 mg: 2 ml).

C. Paper; downward development using n-butanol: acetic acid: water (50:20:30).

Electrophoresis

1. Paper electrophoresis (30 cm) run in ammonium bicarbonate buffer (0.05 M), (pH 8) at current 1 m AMP per cm of paper, voltage 20 V per cm for 2½ hours.

2. Gel electrophoresis by the method of Loening[251], current 5 m AMP per gel.

Spectroscopy

i. Ultraviolet spectra were recorded on Unicam SP 500 or Cary 14 spectrophotometers.

ii. N.M.R. spectra were recorded in D_2O with sodium 2,2-dimethyl-2-silapentane 5-sulphonate (DSS) as internal standard on a Perkin-Elmer R12 instrument at 60MHz. Data at 100 and 220 MHz were obtained from P.C.M.U. Harwell.

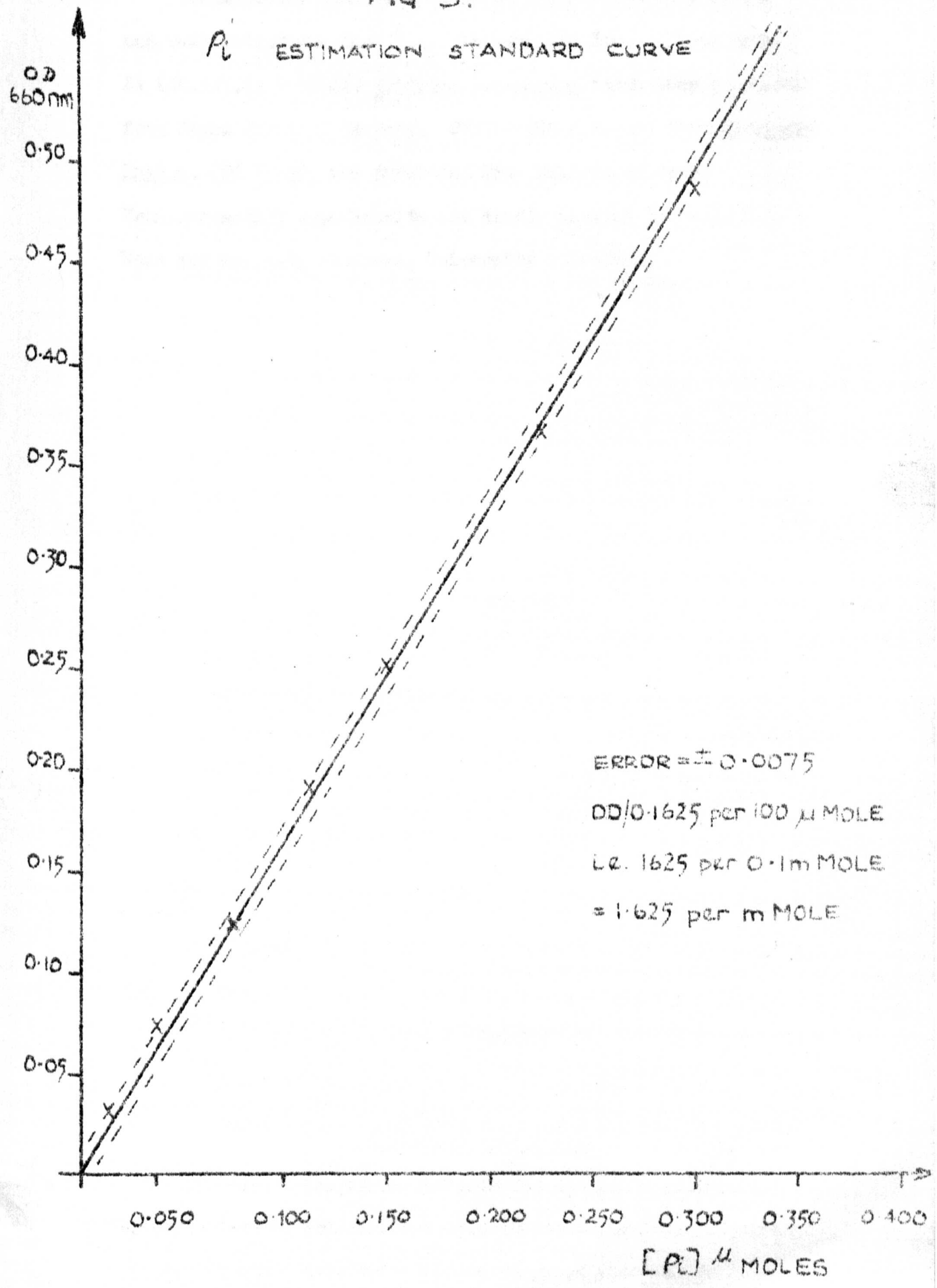
iii. I.R. spectra were recorded on a Perkin-Elmer 257 grating IR spectrophotometer.

iv. C.D. spectra were measured by Dr. P.M. Scopes, Westfield College on a Russel-Jonan dicograph 185 and values expressed in terms of molar ellipticity

Measurement of ϵ (P)

The total phosphorus analysis for the molar extinction co-efficients of polymers was carried out by employing the method of Chen et al[253] to obtain a standard graph (see figure 3).

Fig 3.


Pi ESTIMATION STANDARD CURVE
OD 660nm
0·50
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0·050
0·100
0·150
0·200
0·250
0·300
0·350
0·400
[Pi] μ MOLES
ERROR = ± 0·0075
OD/0·1625 per 100 μ MOLE
i.e. 1625 per 0·1m MOLE
= 1·625 per m MOLE

Materials

Nucleosides and nucleotides were purchased from B.D.H. and polynucleotides from P.L. Biochemicals Inc. PRNase type IA (EC.2.7.7. - 16 and Crotalus adamanteus venum were purchased from Sigma Chemical Company. PNPase (EC.2.7.7.8) from Micrococus luteus, (30 U/mg), was purchased from Boehringer Corp. Trifluoromethyl hypofluorite was kindly donated by Prof. P.W. Kent and Mr. C.G. Butchard, University of Durham.

Chapter One

Poly(5-fluorocytidylic Acid) : Poly($f1^5C$).

N^4,O-2',3',5'-Tetra-acetylcytidine[254].

Acetic anhydride (100 ml) was added to cytidine (5 g) suspended in dry pyridine (200 ml), and the mixture stirred until a clear solution ensued (2 hours). The solution was stirred overnight and then evaporated to dryness in vacuo below 50°C. The residue was dissolved in pyridine (50 ml) which was then evaporated to remove traces of acetic anhydride. After this procedure had been repeated four times, the residue was dissolved in 50% aqueous pyridine (200 ml) and left at room temperature for one hour. Evaporation of this solution to dryness in vacuo gave a gum which was dissolved in ethanol (10 ml), the ethanol being evaporated. This was repeated twice, then the residue was dissolved in ethanol (10 ml) and ether (200 ml) was added to bring down a gummy precipitate. The supernatant was decanted and the residue lyophilised to yield N^4,O-2',3',5',-tetra-acetylcytidine as a powder (4.6 g) (92%). UV pH 1 λmax 240 nm, ε 7,800, 307 nm, ε 6,600; pH 6 λmax 248 nm ε 6,200, 297 nm ε 6,000; pH 12 λ max 275 nm (sh) ε 6,100, 303 nm ε 5,600.

^{1}HNMR (220 MHz), τ1.9 (1H d) (J = 7.2 Hz), (H^5), τ 2.1 (1H d) (J = 7.2 Hz) (H^6), τ 3.9 (1H d), (J = 3.6 Hz) ($H^{1'}$), τ 4.5 (2 H m), ($H^{2'}$,$H^{3'}$) τ 5.5 (3 H d) (J = 2.4 Hz) ($H_2^{5'}$), τ7.8 (12 H s) (CH_3CO).

This product was used for the next stage without further purification.

N^4,O-2',3',5'-Tetra-acetyl 5-fluorocytidine.

N^4,O-2',3',5'-tetra-acetyl cytidine (4.5 g) was dissolved in trichlorofluoromethane/chloroform (75 ml/25 ml) and the mixture cooled with stirring in dry ice/acetone bath until the temperature had fallen to -80°C. The system was flushed out with dry nitrogen and then trifluoromethane hypofluorite was slowly passed into the

solution. After 1.5 g of trifluoromethyl hypofluorite was passed through the system over three hours, the supply of trifluoromethyl hypofluorite was stopped and stirring at -78°C continued for another hour. The dry ice/acetone bath was then removed and the temperature was allowed to rise gradually to room temperature (1.5 hours). Nitrogen was bubbled through the system to remove any traces of trifluoromethyl hypofluorite in solution (1 hour) when most of the solvent evaporated. The residue was dissolved in methanol (80 ml) and triethylamine (20 ml) added. The solution was heated under reflux at 70°C (30 min) after which the solution was evaporated to dryness and the residue purified using Dowex 50 (H^+ resin) with gradient elution by 0.001 N to 0.1 N ammonium hydroxide. The 5-fluorocytidine obtained was crystallised from water : ethanol : diethyl ether (1 : 50 : 50) to yield (3.2 g) 68%. Analysis calculated for $C_9H_{12}O_5N_3F$: C, 41.38; H, 4.63; N, 16.08; F, 7.27%, found C, 41.59; H, 4.77; N, 16.23; F, 7.23%. Melting point 220 - 222°C. UV pH 2 λmax 289 nm, ε 6,800, pH 7 λmax 281 nm, ε 6,400; pH 12 λmax 279 nm ε 6,300.

^{1}HNMR (220 MHz): τ 1.98 (1 Hd), (J = 7.2 Hz), (H^6 split by F^5); τ 4.15, (1 Hd), (J = 2.1 Hz) ($H^{1'}$), τ 5.8 - 6.2, (5 Hm), (sugar protons).

2',3'-O-Isopropylidene-5-fluorocytidine[255].

5-fluorocytidine (100 mg) was dissolved in acetone (10 ml) containing p-toluenesulphonic acid (1 g) and the solution stirred at room temperature (30 min). Sodium bicarbonate (1 g) was added and stirring continued (1 hour). The precipitate was filtered and washed twice with hot acetone (10 ml) and all filtrates were combined and evaporated to dryness. Recrystallisation was either from acetone-hexane (50:50) or hot water to yield 48 mg (45%). This crude material was used for the next stage without further purification. UV pH 1 λmax 284 nm, ε 6,200; pH 7 λmax 273 nm, ε 6,050, λ 258(sh)

ε 5,900; pH 11 λ max 273, ε 6,000 , λ 238 (sh), ε 5,800.

^{1}H NMR (60 MHz):- τ 1.98 (1 Hd), (J = 7.2 Hz), (H^6 split by F^5); τ 4.15 (1 Hd), (J = 2.1 Hz) ($H^{1'}$), τ 5.8 - 6.2 (5 Hm), (sugar protons), τ 6.8 (6 Hd) (J = 7.2 Hz), Isopropylidene protons.

5-Fluorocytidine 5'-monophosphate (fl^5 CMP)[256].

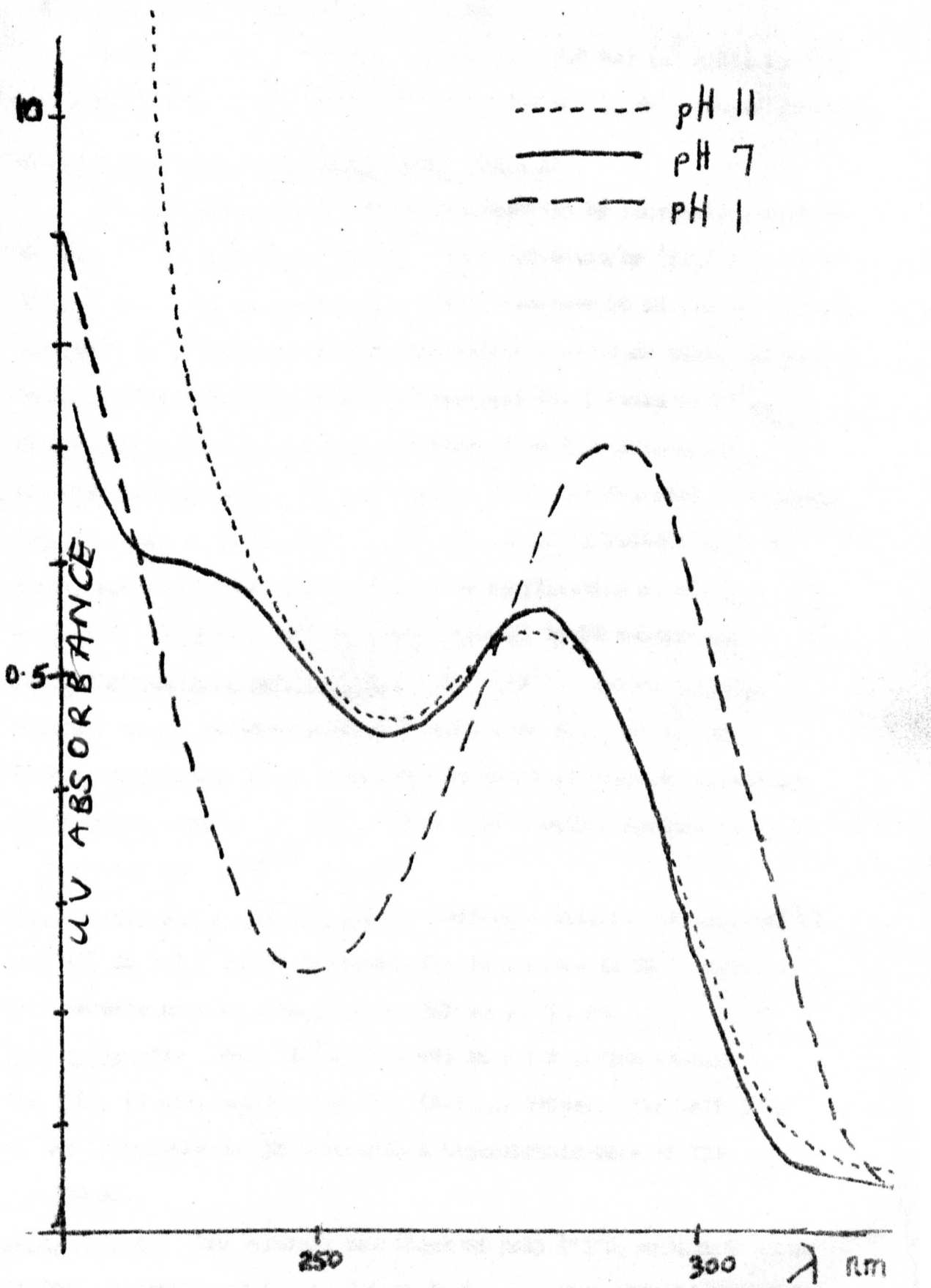
5-fluorocytidine (500 mg) was dissolved in trimethyl phosphate (5 ml) and cooled to -40°C in an acetone/dry ice bath when a solution of phosphoryl chloride (0.6 - 0.9 ml) in trimethyl phosphate (10 ml) at -40°C) was added dropwise. After complete addition, the mixture, vigorously stirred was allowed to warm up to -5°C in an ice/salt bath. The solution was then maintained at this temperature for 4 hours when it was allowed to warm up gradually to room temperature. Water (20 ml) was added and trimethyl phosphate was extracted from the solution by diethyl ether (30 ml x 3). The aqueous layer was then evaporated to dryness and the residue dissolved in water (50 ml) containing ammonium hydroxide (50 ml). The solution was heated for 30 minutes at 70°C to decompose any phosphochloridates. The solution was evaporated to dryness, the residue dissolved in water and passed down a Dowex 50 (H^+) ion exchange resin column with elution by water during which the isopropylidene group was removed and chromatographically pure 5-fluorocytidine 5'-monophosphate was obtained with a yield of 200 mg (40%), M.P. 232 - 234°C. UV (fig. 4) pH 2 λ max 289 nm ε 6,600, pH 7 λ max 279 nm ε 6,000, pH 12 λ max 279 nm, ε 6,200. ^{1}H NMR (60 MHz):- τ 1.98 (1 Hd), (J = 7.2 Hz), (H^6 split by F^5); τ 4.15 (1 Hd), (J = 2.2 Hz), ($H^{1'}$) τ 5.6 - 6.2 (5 Hm), (sugar protons). The isopropylidine signal, has disappeared.

5-Fluorocytidine 5'-diphosphate (fl^5 CDP).

5-fluorocytidine 5'-diphosphate was prepared from 5-fluorocytidine 5'-monophosphate (100 mg) by the method of Moffat and Khorana[257] to yield 40 mg 38%, chromatographically pure and electrophoretically homogeneous. Rf on system D was 0.34 as compared to the

FIG 4.

UV SPECTRA OF 5-FLUOROCYTIDINE 5'-MONOPHOSPHATE



Rf of 0.56 for 5-fluorocytidine 5'-monophosphate. Electrophoretic mobility is 0.74 as compared to 0.42 for the 5-fluorocytidine 5'-monophosphate. UV pH 2 λ max 290 nm ε 6,800, pH 7 λmax 281 nm ε 6,500, pH 11 λ max 280 nm ε 6,200.

^{1}HNMR (60 MHz): τ 1.99, (1 Hd), (J = 7.0 Hz) (H^6 split by F^5), 4.15 (1 Hd), (J = 2.0 Hz) $H^{1'}$, τ 5.6 - 6.2 (5 Hm), (sugar protons).

Poly (5-fluorocytidylic acid), (poly (fl^5C)):

The polymerisation of fl^5CDP was followed by inorganic phosphate release [258] to give Km 8.8 mM and Vmax 1.0 /umole/hr (fig. 5). In a typical case, the reaction medium which contained 20 mM (10 mg) fl^5CDP, 1.2 mg/ml of PNPase, 10 mM magnesium chloride and 5 mM NaEDTA in 0.15 M tris-chloride buffer (pH 9.0) was incubated for 8 hours at 37°C. After deproteinisation by repeated extraction with chloroform/ isoamyl alcohol (5:2, v/v); the aqueous phase was desalted by dialysis over 36 hours at 5°C against 0.1 M NaCl - 0.001 M NaEDTA; 0.001 M NaEDTA and then twice against water. Lyophilisation at 0°C gave poly (fl^5C) (4.5 mg), 46% yield as estimated by UV absorption.

Characterisation of poly (fl^5C):- Poly (fl^5C) ran as a single compound on gel electrophoresis [251] and had an $S_{20,W}$ of 6.6 by ultracentrifugation in an isokinetic gradient of sucrose containing 0.1 M Sodium acetate (pH 7.5). UV in 0.01 M sodium acetate (pH 7.0) λmax 280 nm ϵ(P) [188] = 5,800.

Sodium hydroxide digest (fig. 6):- Heating a solution of poly (fl^5C) (0.2 /uM) in 0.1 N sodium hydroxide for 15 minutes at 90°C caused a hyperchromic rise in absorption of 14% at λ 280 nm.

PRNase digest:- Poly (fl^5C) (0.2/uM) in 0.1 M sodium acetate (pH 7.0), (2 ml), was treated with (0.5 /ug) PRNase. The half life of the hydrolysis was 36 sec. with a hyperchromic rise of 13% λ 280 nm.

pH Titration:- Two separate solutions of poly (fl^5C) were made with an optical density of 0.5 at 290 nm in 0.1 N sodium chloride(a) pH 7

FIG. 5.

POLYMERISATION KINETICS OF 5-FLUOROCYTIDINE DIPHOSPHATE.

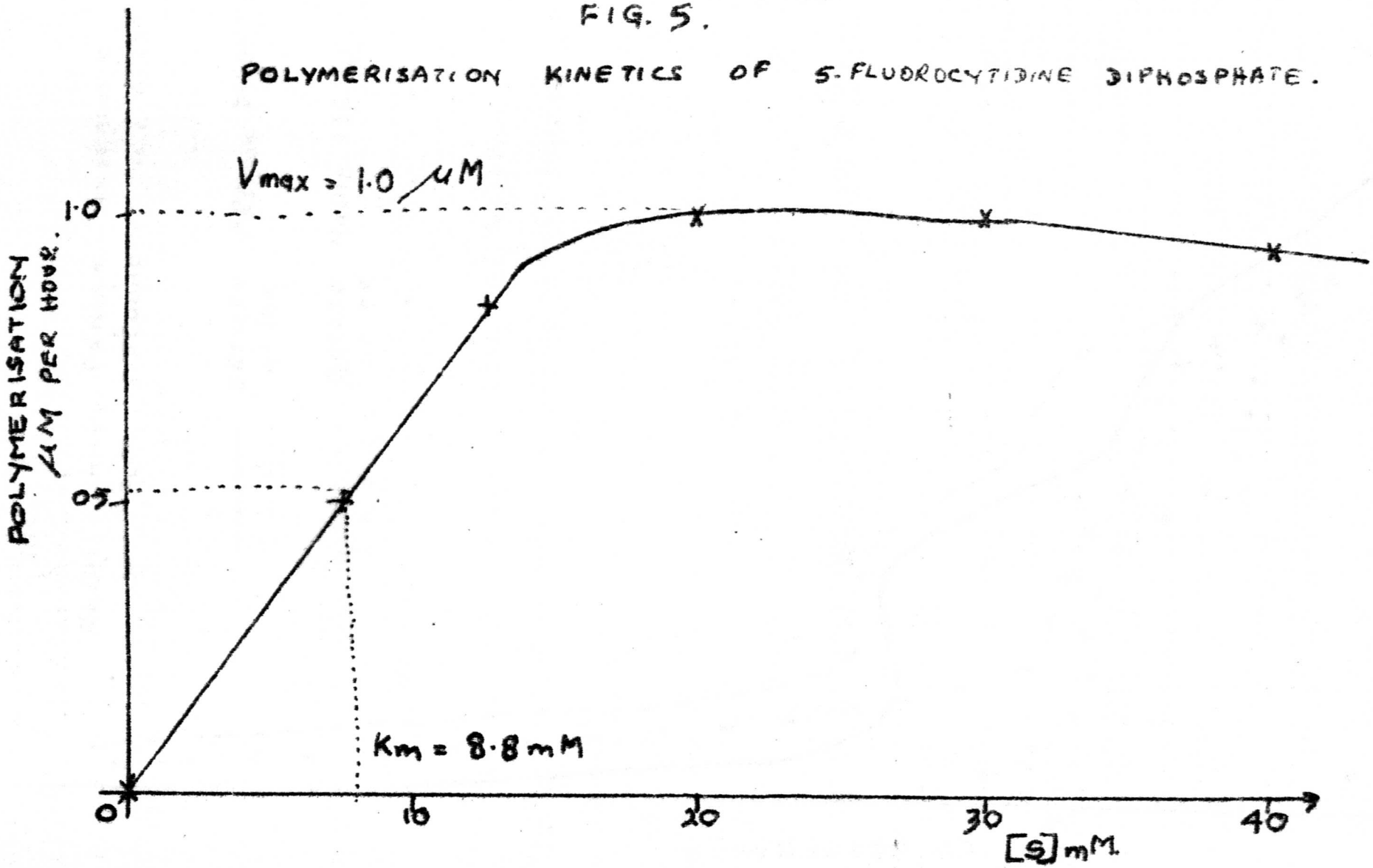
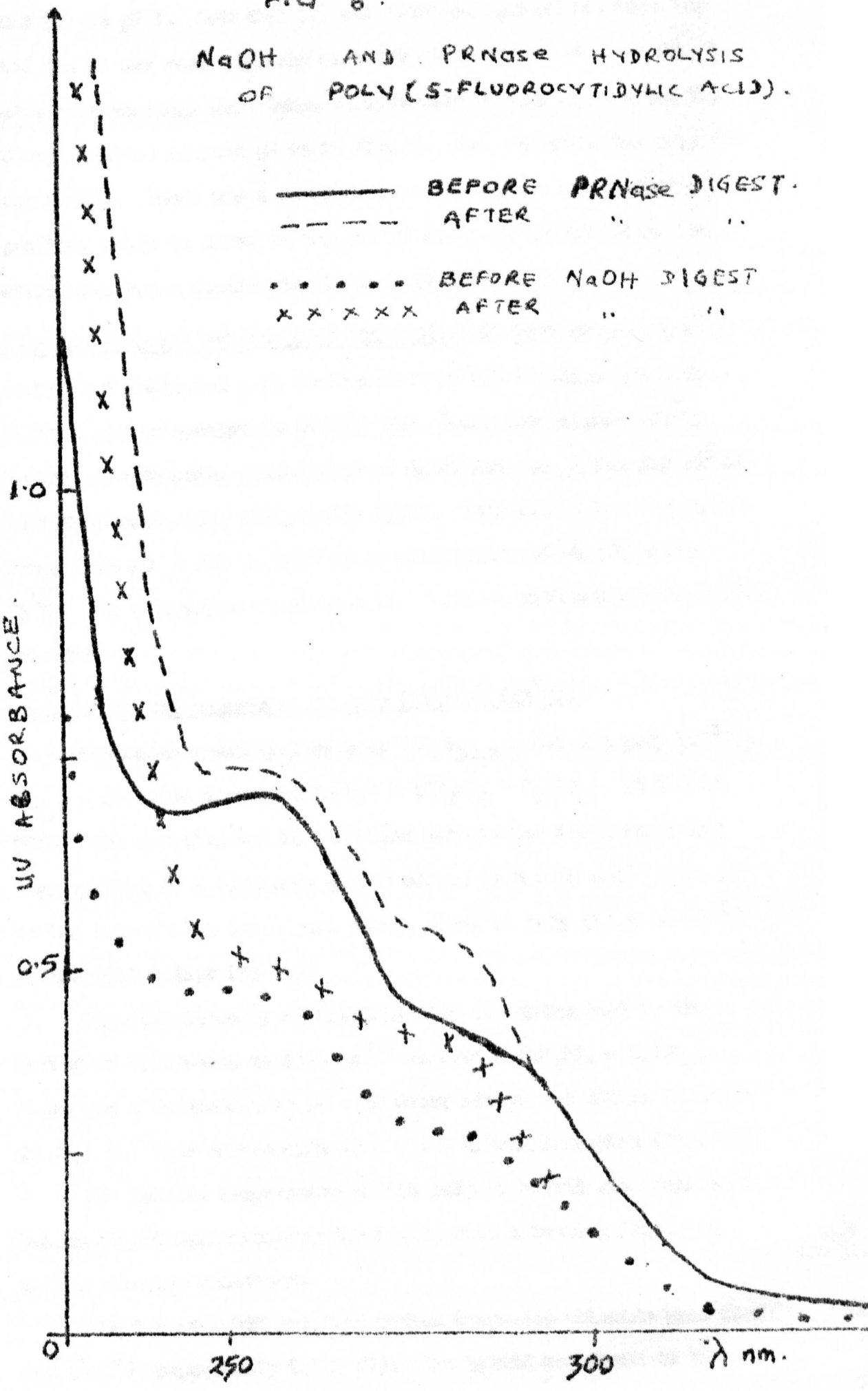


FIG 6.

NaOH AND PRNase HYDROLYSIS OF POLY (5-FLUOROCYTIDYLIC ACID).



and (b) at pH 1. Solution (b) was titrated against solution (a) and the pH was read together with the OD at 290 nm at that pH. A series of readings were taken at intervals of 0.2 pH unit and the OD was plotted against pH as in Fig. 7. The procedure was repeated for $f1^5$CDP. There was a sharp rise at pH 2.45 in the polymer profile, which is close to the pKa of 2.42; $f1^5$CDP exhibiting no sharp rise but a gentle rise between pH 1.5 and 3.

Circular dichroic spectra (CD) (Fig. 8):- CD both of poly ($f1^5$C) and $f1^5$CDP indicated anti conformation in 0.3 N sodium chloride - 0.001 N sodium cacodylate pH 7. The λmax for polymer ($f1^5$C) is 280 nm with molar ellipticity of 4,500 and the λ max for $f1^5$CDP is 270 nm with molar ellipticity 3,500. Poly ($f1^5$C) has a negative absorption at λ 218 nm with molar ellipticity of -1,500 while $f1^5$CDP has a negative absorption at λ 215 nm with molar ellipticity of - 900.

Preparation and properties of poly (I).poly ($f1^5$C):-

Equimolar quantities of poly (I) $S_{20,w}$ = 6.5 and poly ($f1^5$C), $S_{20,w}$ = 6.6 were dissolved in 0.1 M (KH_2PO_4 + K_2HPO_4), pH 7.0 at 37°C. The solution was left at room temperature for 2 hours and then applied to a Sepharose 4B 200 column (1.5 x 30 cm). Elution of the column with water gave double stranded poly (I).poly ($f1^5$C) in the void volume (35 ml).

The stoichiometry of hybridisation was determined by the method of continuous variations[259] in 0.01 M (KH_2PO_4 + K_2HPO_4). There was a deflection at 50 : 50 molar mixture at 245 nm as shown in fig. 9. This corresponds to a 1 : 1 hybrid formation (fig. 10).

The melting temperature of the polymer hybrid was found on a Unicam SP 500 spectrophotometer fitted with a heating block, in sodium chloride solutions.

The Tms in 0.01N and 0.1N sodium ~~(fig. 11)~~ chloride were 60.5°C and 66.5°C respectively (fig. 11). The hybrid was found to be a good inducer of interferon production (see appendix).

Fig. 7.

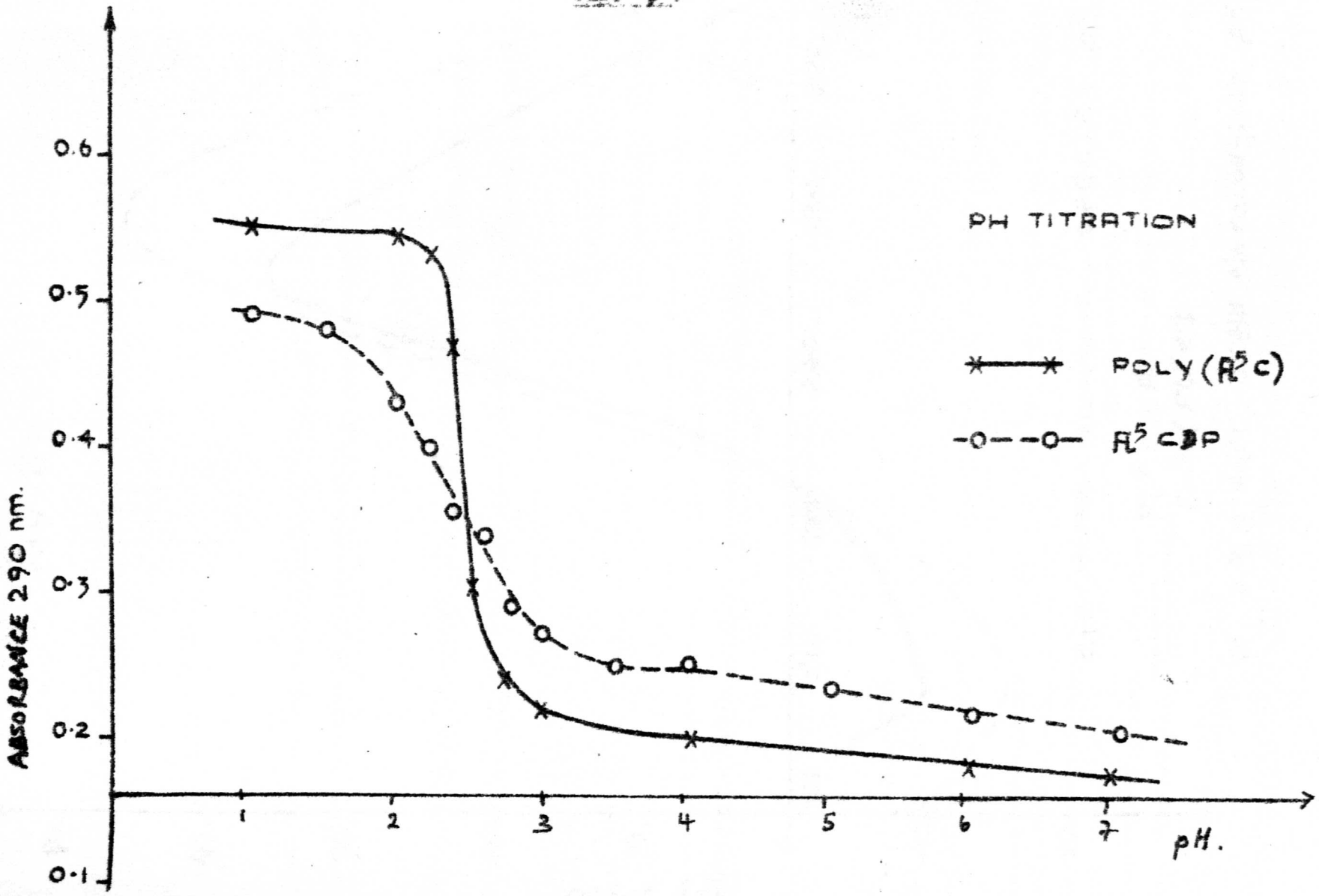
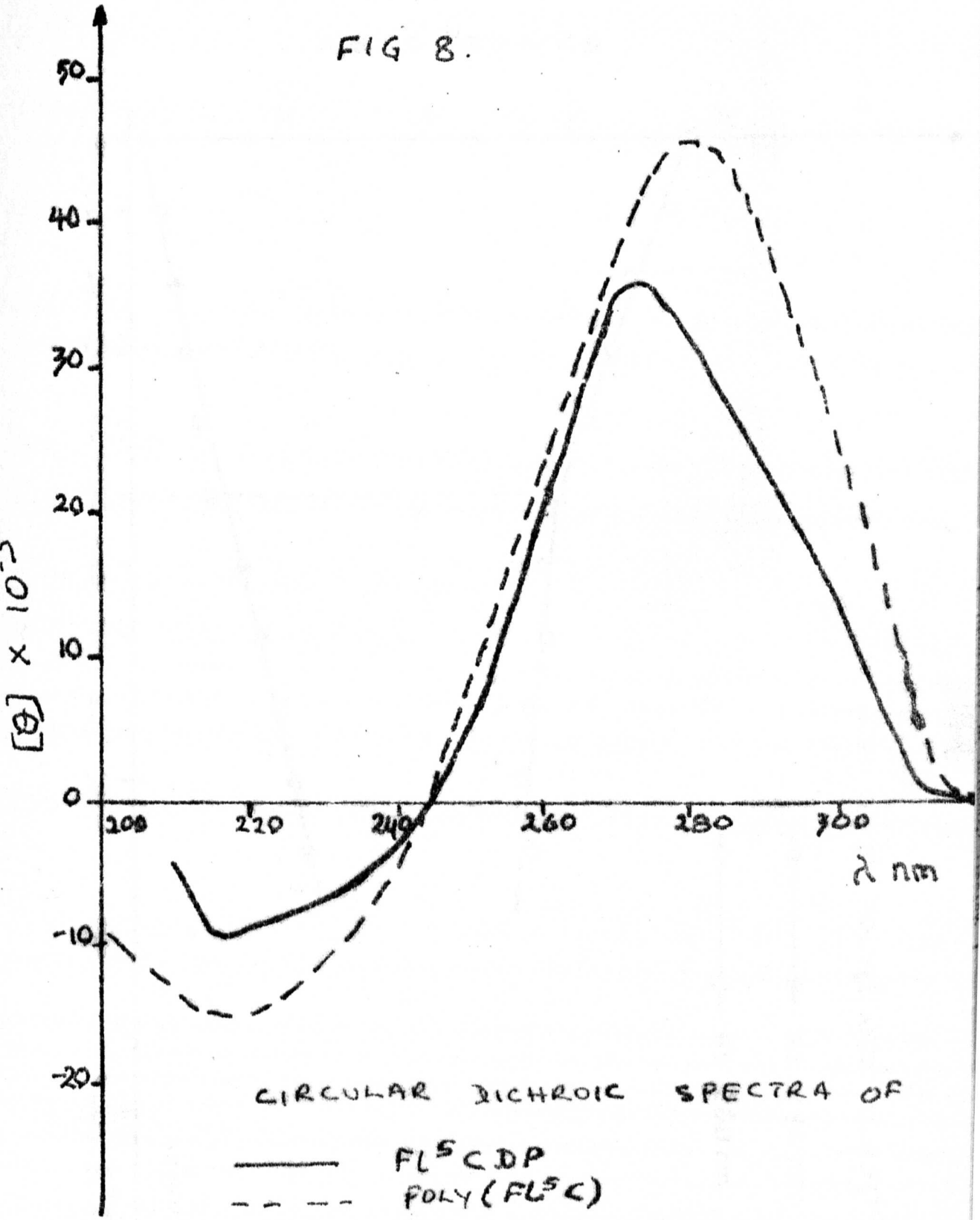

PH TITRATION
POLY (R^5C)
R^5CDP
ABSORBANCE 290 nm.
0·6
0·5
0·4
0·3
0·2
0·1
1
2
3
4
5
6
7
pH.

FIG 8.



CIRCULAR DICHROIC SPECTRA OF

——— FL^5CDP

– – – – POLY(FL^5C)

IN 0·3 N NaCl, 0·001N Na cacodylate, pH 7·0

FIG 9.

MIXING OF POLY(I) AND POLY(Fl^5C)

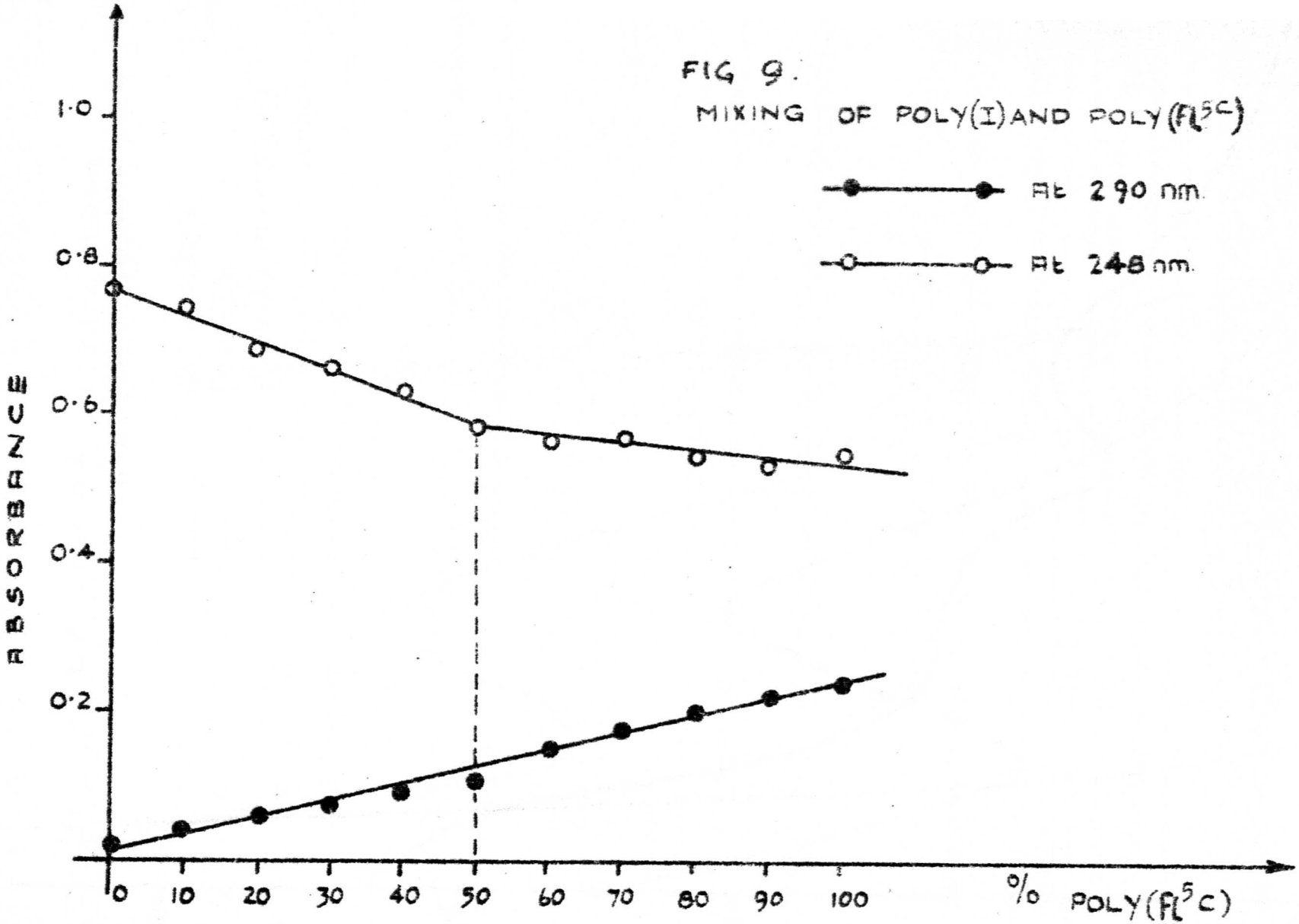


FIG 10.

UV SPECTRA IN 0·1M SODIUM CACODYLATE BUFFER, pH 7·0

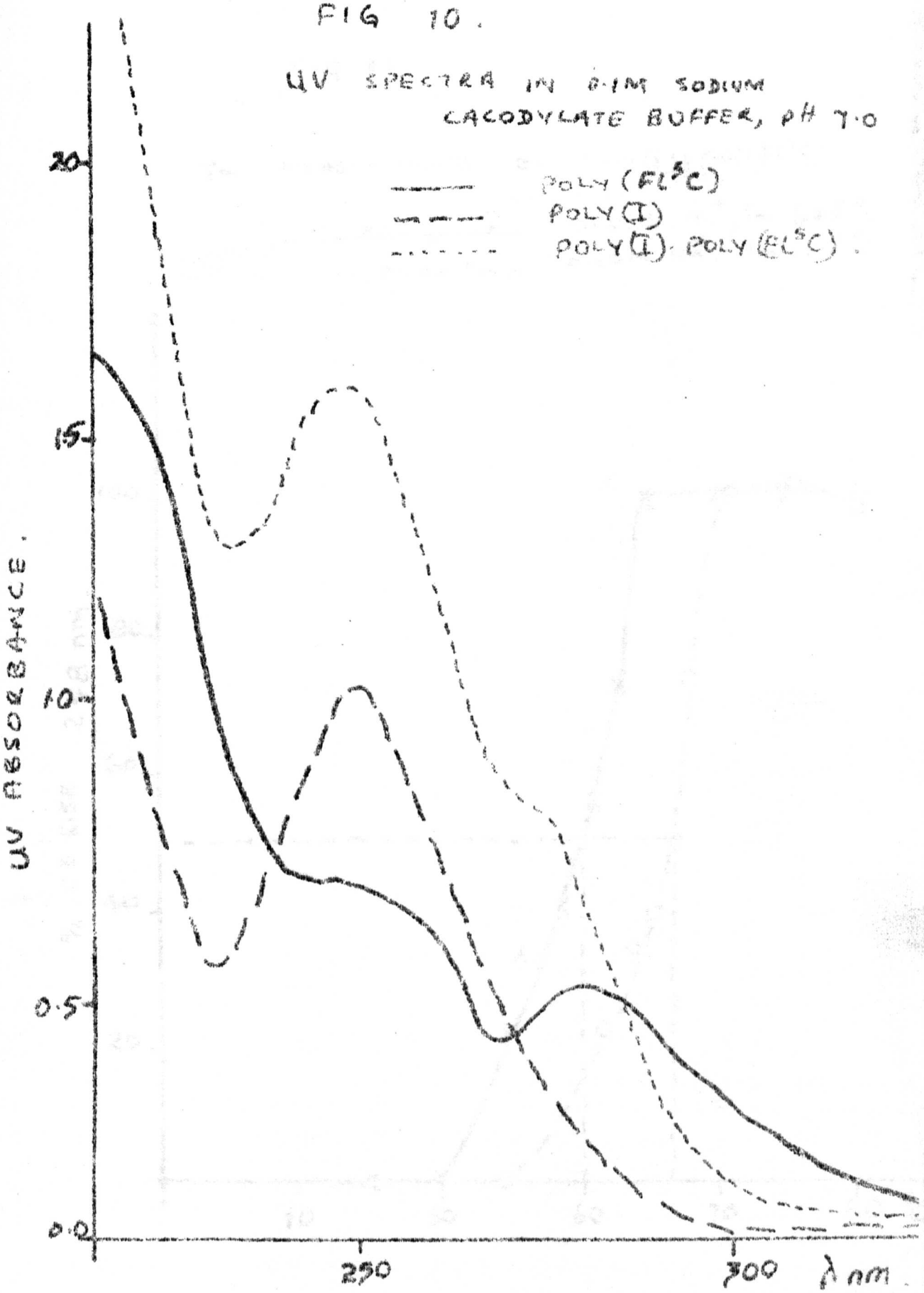
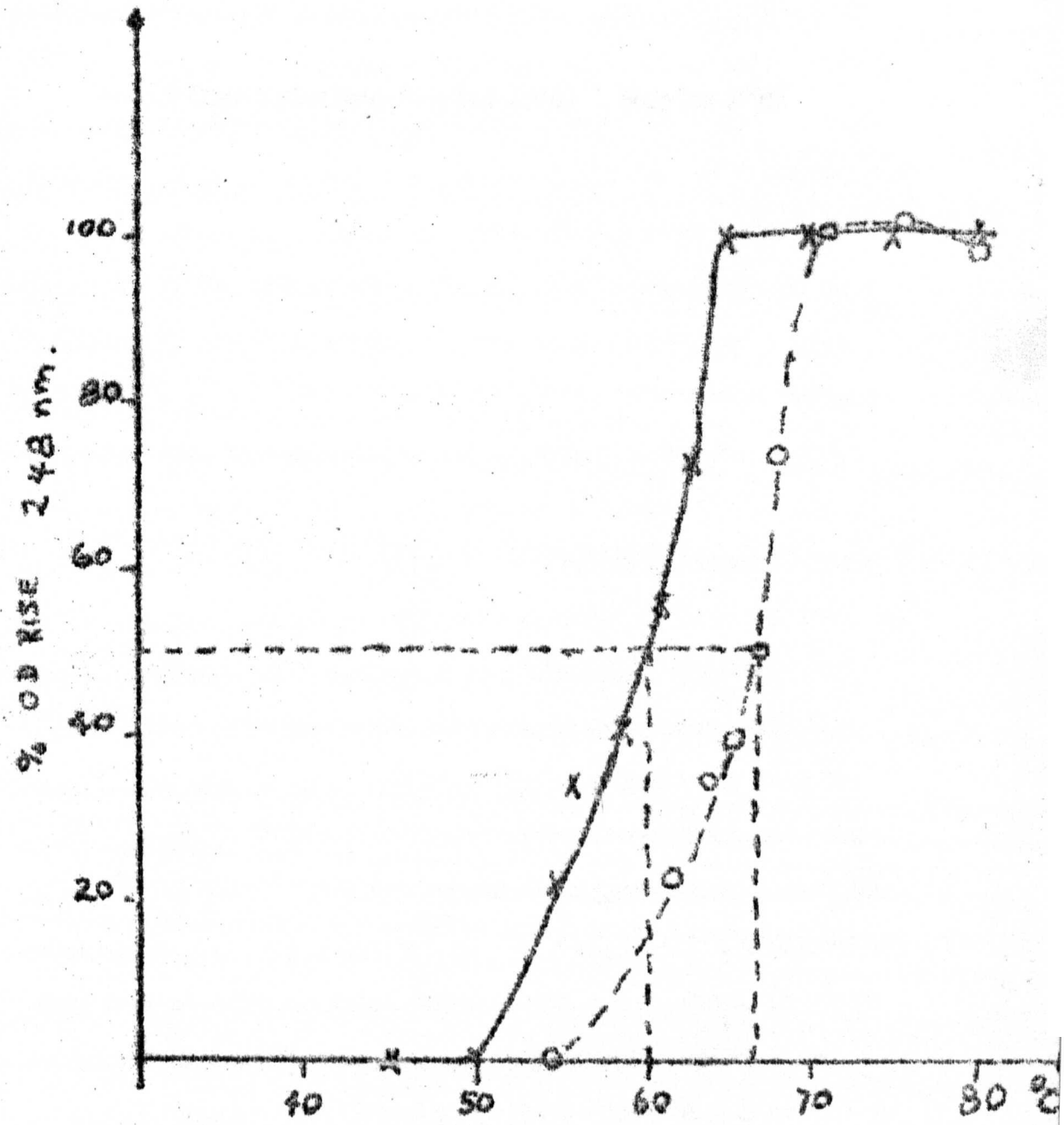


FIG 11.

Tm MEASUREMENTS OF POLY(I) . POLY(FI^5C).

—x—x— 0.01M Na^+, Tm 60.5 °C.

--o--o-- 0.10M Na^+, Tm 66.5 °C.



Chapter Two

Poly(5-Dimethylaminocytidylic Acid) : Poly(Me_2N^5C).

5-Dimethylaminocytidine 5'-monophosphate[245].

5-Bromocytidine 5'-monophosphate[228] (1 g) was converted into the free acid using Dowex 50 (H^+) ion exchange resin (1 cm x 30 cm) and was dissolved in water (20 ml) when the pH was brought to 7.0 by adding tetrabutylammonium hydroxide. This was evaporated to dryness and left on an oil pump until completely anhydrous, and the residue was dissolved in dry DMF (10 ml) with warming. Anhydrous dimethylamine (3 ml) previously cooled to 0°C was added. The reaction was heated with stirring on a water bath at 60°C for 8 hours under an acetone-dry ice-cooled condenser. More dimethylamine (3 ml) was added at intervals of 2 hours. The flask was connected to a water condenser and further heated overnight to let excess dimethylamine evaporate gradually and to allow for more reaction. The reaction mixture was poured into a beaker and water (50 ml) was added. The pH was brought to 2 with HCl, the solution was evaporated to dryness and the residue was purified by chromatography on Dowex 50 (H^+) ion exchange resin (1 cm x 30 cm). If the reaction mixture during reflux was not completely anhydrous 5-hydroxycytidine 5'-monophosphate was formed as a byproduct and this was eluted from the column first. The unreacted 5-bromocytidine 5'-monophosphate came in the second peak, and finally, well separated from the other compounds, 5-dimethylaminocytidine 5'-monophosphate came in the third peak. When the reaction mixture during reflux was completely anhydrous there was no 5-hydroxycytidine 5'-monophosphate formed. The reaction never went to completion, so there was always unreacted 5-bromocytidine 5'-monophosphate. The 5-dimethylaminocytidine 5'-monophosphate peak was evaporated to dryness to yield 350 mg (32%), chromatographically pure. Calculated analysis for $C_{11}H_{19}O_8N_4P.H_2O$, C, 34.38; H, 5.50; N, 14.57, P, 8.06 % found C, 34.34; H, 5.60; N, 14.41; P, 8.20 %. UV pH 1 λ max 312 nm ε 4,800, 218 nm ε 9,000, pH 7 λ max

294 nm ε 6,000, 224 nm ε 8,500, pH 12 λ max 294 nm ε 7,000, 224 nm ε 20,000.

^{1}HNMR (100 MHz) τ 2.23, (1Hs), (H^6); τ 4.1, (1Hd), (J = 3.0 Hz), ($H^{1'}$); τ 5.0 - 6.0, (5H m), sugar protons; τ 7.35, (6H s), Me_2N.

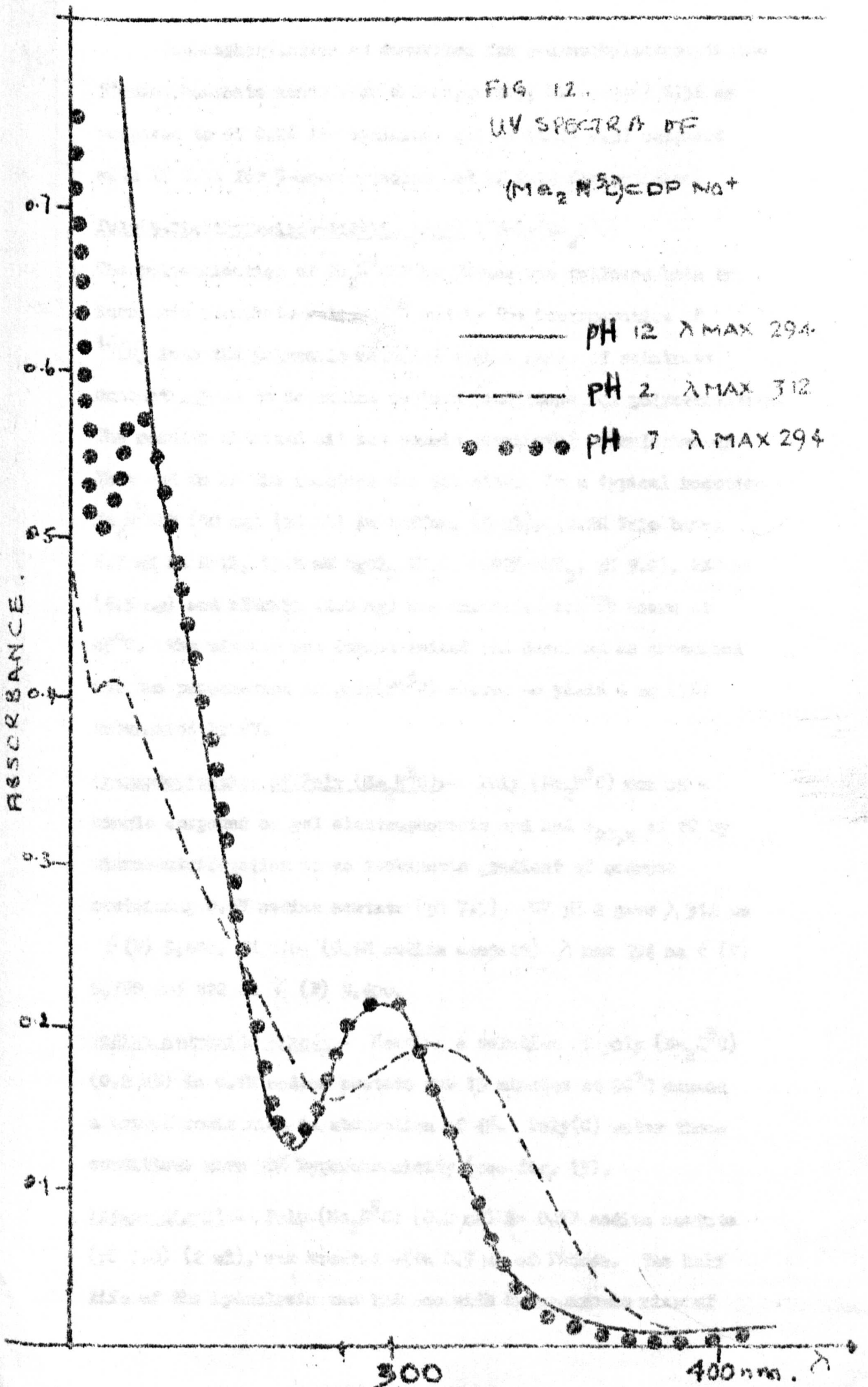
Dephosphorylation[260]. 5-Dimethylamino cytidine 5'-monophosphate (10 μM) in 0.1M Tris acetate buffer (1 ml, pH 8.0) was incubated with Crotalus adamanteus venom (100 μg) for 12 hours at 37°C. Examination of the reaction mixture by paper (system H) and TLC (system A) showed only one compound with Rf (paper) 0.36 compared with Rf 0.24 for cytidine and Rf (TLC) 0.37 compared with Rf 0.35 for 5-bromocytidine and 0.33 for cytidine.

pKa determination: By a similar method as described in the pH titration of poly($f1^5C$) and $f1^5$ CDP above pKa was found to be 4.14 ± 0.07 at 22°C.

5-Dimethylaminocytidine 5'-diphosphate:- Me_2N^5CDP, was prepared from 5-dimethylaminocytidine 5'-monophosphate (100 mg) as described by Moffat and Khorana[257] to yield 36 mg, 32%, chromatographically pure and electrophoretically homogeneous. Rf on system D was 0.42 as compared to the Rf of 5-dimethyl aminocytidine 5'-monophosphate of 0.65 and electrophoretic mobility of 0.62 as compared to the electrophoretic mobility of 5-dimethylaminocytidine 5'-monophosphate of 0.36. Analysis calculated for $C_{11}H_{17}O_{11}N_4P_2$, C,25.79; H,3.34; N,10.93; P,12.09 %, found C,26.47; H,4.46; N,10.14; P,11.33 %. UV pH 1 λ max 312 nm ε 4,900; 218 nm ε 9,500, pH 7 λ max 294 nm ε 6,200, 224 nm ε 9,000; pH 12 λ max 294 nm ε 6,900, 224 nm ε 18,000. (See Fig. 12.) ^{1}HNMR (100 MHz); τ 2.24, (1Hs), (H^6); τ 4.1 (1Hd), (J = 3.2 Hz), ($H^{1'}$); τ 5.0 - 6.2, (5H m) sugar protons; τ 7.35, (6H S), Me_2N. ^{14}[C] -labelled Me_2N^5CDP specific activity 180 dpm/μmole was prepared similarly.

FIG 12.
UV SPECTRA OF

(Me_2NSO)=DP Na^+



Dephosphorylation as described for 5-dimethylaminocytidine 5'-monophosphate above showed homogeneity, Rf (paper) 0.36 as compared to Rf 0.26 for cytidine, and Rf (TLC) 0.37 compared with Rf 0.34 for 5-bromocytidine and Rf 0.32 for cytidine.

Poly(5-Dimethylaminocytidylic Acid) : Poly(Me_2N^5C).

The polymerisation of Me_2N^5CDP by PNPase was followed both by inorganic phosphate release[258] and by the incorporation of $^{14}[C]$ into the polymeric material over a range of substrate concentrations to determine optimum conditions for polymerisation. The results obtained did not permit meaningful calculation of Vmax and Km as the reaction was too slow. In a typical reaction Me_2N^5CDP (50 mg) (20 mM) in buffer, (5 ml), (0.2M Tris base, 6.7 mM Na EDTA, 13.3 mM $MgCl_2.6H_2O$, 0.02% NaN_3, pH 9.0), PNPase (6.5 mg) and albumen (2.0 mg) was incubated for 60 hours at 45°C. The mixture was deproteinised and desalted as described for the preparation of poly(fl^5C) above, to yield 4 mg (7%) determined by UV.

Characterisation of Poly (Me_2N^5C):- Poly (Me_2N^5C) ran as a single compound on gel electrophoresis and had $S_{20,w}$ of 10 by ultracentrifugation in an isokinetic gradient of sucrose containing 0.1M sodium acetate (pH 7.5). UV pH 2 gave λ 312 nm ϵ (P) 5,400, pH 7.0, (0.1M sodium acetate) λ max 294 nm ϵ (P) 5,700 and 222 nm ϵ (P) 5,400.

Sodium hydroxide digest:- Heating a solution of poly (Me_2N^5C) (0.2 μM) in 0.1M sodium acetate for 15 minutes at 90°C caused a hyperchromic rise in absorption of 4%. Poly(C) under these conditions gave 30% hyperchromicity (see fig. 13).

PRNase digest:- Poly (Me_2N^5C) (0.2 μM) in 0.1M sodium acetate (pH 7.0) (2 ml), was treated with 0.5 μg of PRNase. The half life of the hydrolysis was 124 sec with hyperchromic rise of

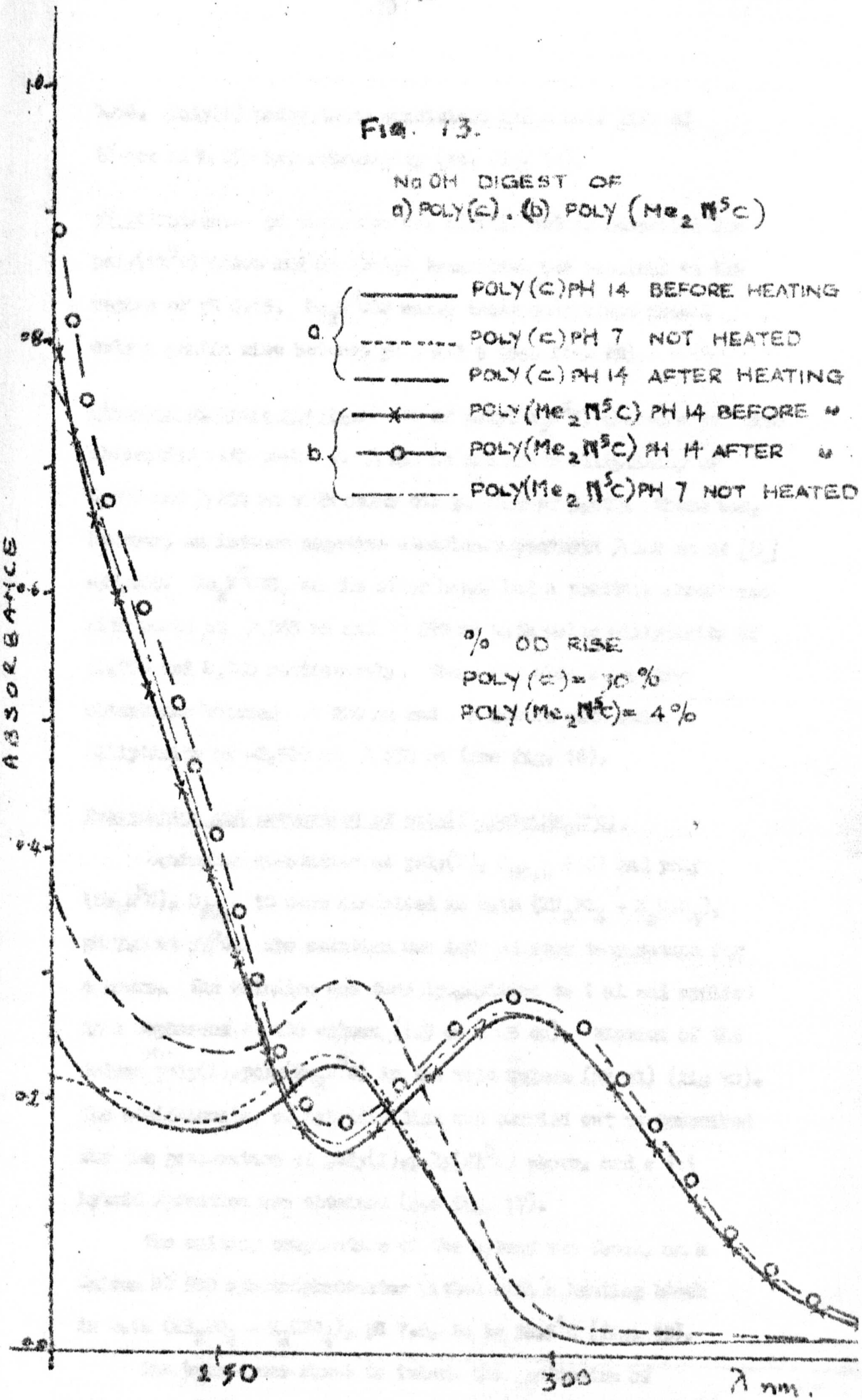


Fig. 13.
NaOH DIGEST OF
a) POLY(C). (b) POLY (Me_2 N^5C)
a. POLY(C) PH 14 BEFORE HEATING
POLY(C) PH 7 NOT HEATED
POLY(C) PH 14 AFTER HEATING
b. POLY(Me_2 N^5C) PH 14 BEFORE "
POLY(Me_2 N^5C) PH 14 AFTER "
POLY(Me_2 N^5C) PH 7 NOT HEATED
% OD RISE
POLY(C) = 30 %
POLY(Me_2 N^5C) = 4 %
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0.0
250
300
λ nm.

3.5%. Poly(C) under these conditions had a half life of 12 sec with 25% hyperchromicity (see Fig. 14).

pH Titration:- pH titration was carried out as described for poly(fl^5C) above and an abrupt transition was obtained in the region of pH 4.15. Me_2N^5CDP under these conditions showed only a gentle rise between pH 3 and 5 (see fig. 15).

Circular dichroic spectra:- CD of poly(Me_2N^5C) showed a positive absorption with peaks at λ 255 nm and molar ellipticity of 5,000 and λ 294 nm with molar ellipticity of 3,000. There was, however, an intense negative absorbance around λ 220 nm of $[\theta]$ -37,000. Me_2N^5CDP, on the other hand, had a positive absorbance with peaks at λ 245 nm and λ 290 nm with molar ellipticity of 10,000 and 8,000 respectively. There was also a negative absorbance between λ 220 nm and λ 240 nm with molar ellipticity of -2,500 at λ 230 nm (see fig. 16).

Preparation and properties of poly(I).poly(Me_2N^5C).

Equimolar quantities of poly(I), $S_{20,w}$ 6.64 and poly (Me_2N^5C), $S_{20,w}$ 10 were dissolved in 0.1M (KH_2PO_4 + K_2HPO_4), pH 7.0 at 37°C. The solution was left at room temperature for 4 hours. The solution was then lyophylised to 1 ml and applied to a Sepharose 4B 200 column (1.5 cm x 25 cm). Elution of the column gave poly(I).poly(Me_2N^5C) in the void volume (28 ml) (fig 18). The stoichiometry of hybridisation was carried out as described for the preparation of poly(I).poly(fl^5C) above, and a 1:1 hybrid formation was obtained (see fig. 17).

The melting temperature of the hybrid was found, on a Unicam SP 500 spectrophotometer fitted with a heating block in 0.1M (KH_2PO_4 + K_2HPO_4), pH 7.0, to be 58.5°C (fig. 19).

The hybrid was found to induce the production of interferon (see appendix).

FIG 14.

PRNASE DIGEST OF
1. POLY (Me_2n^5c)

——— SOON AFTER ADDING ENZYNE

- - - - - AFTER INFINITY REACTION

2. POLY (c)

—×—×— BEFORE DIGEST

—o—o— AFTER DIGEST

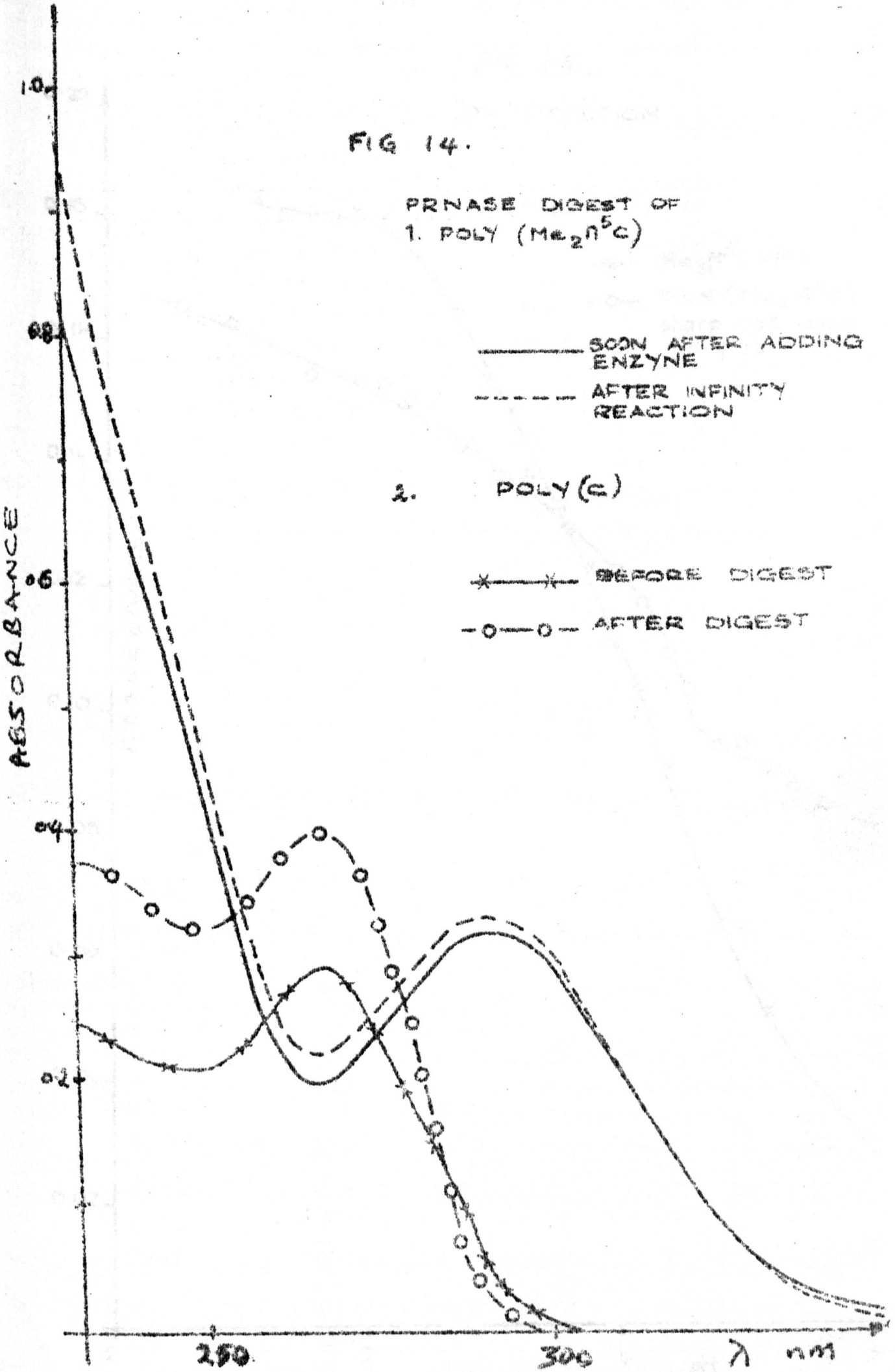


FIG 15.

PH TITRATION

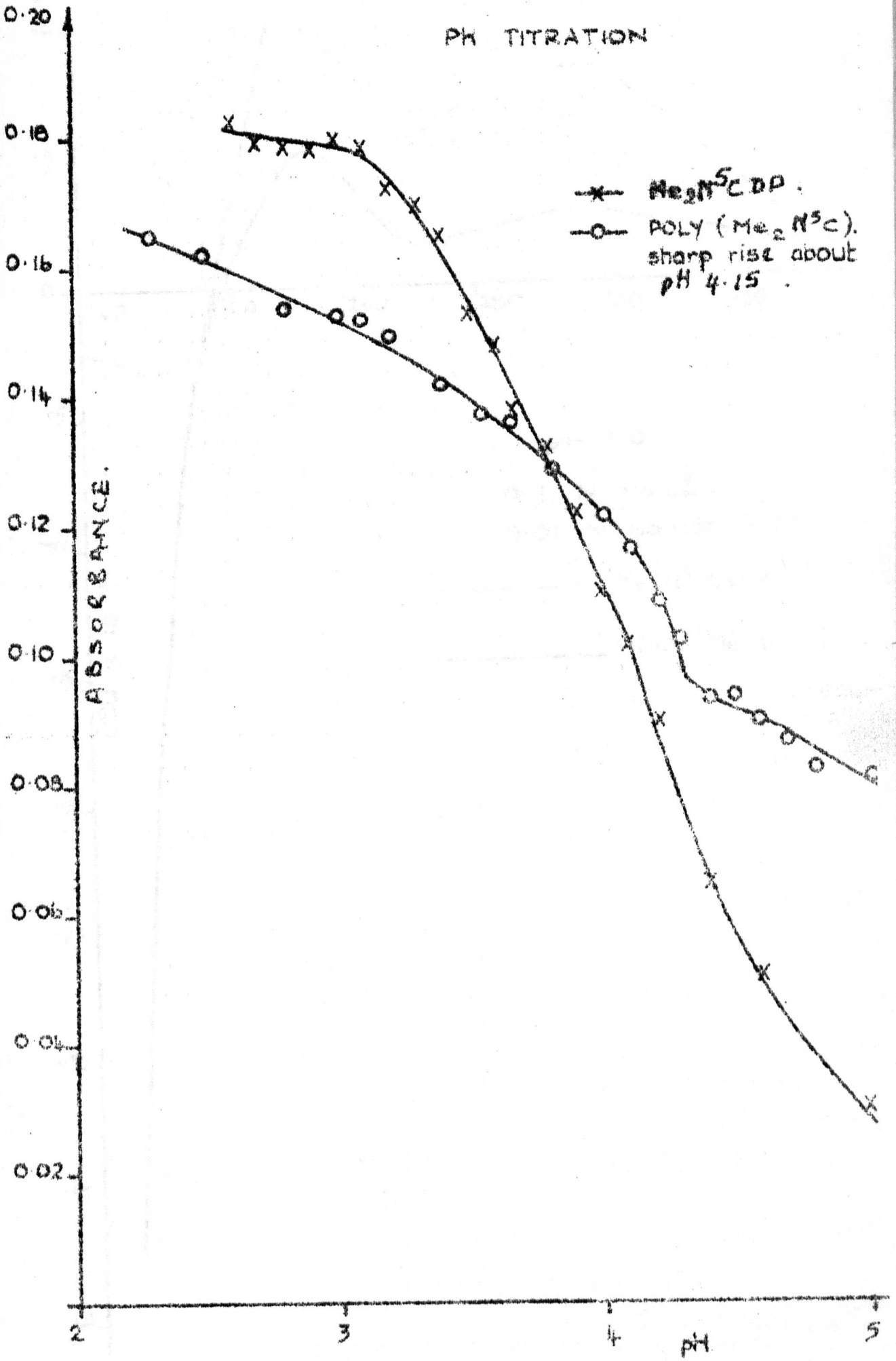


FIG 16.

CIRCULAR DICHROIC SPECTRA

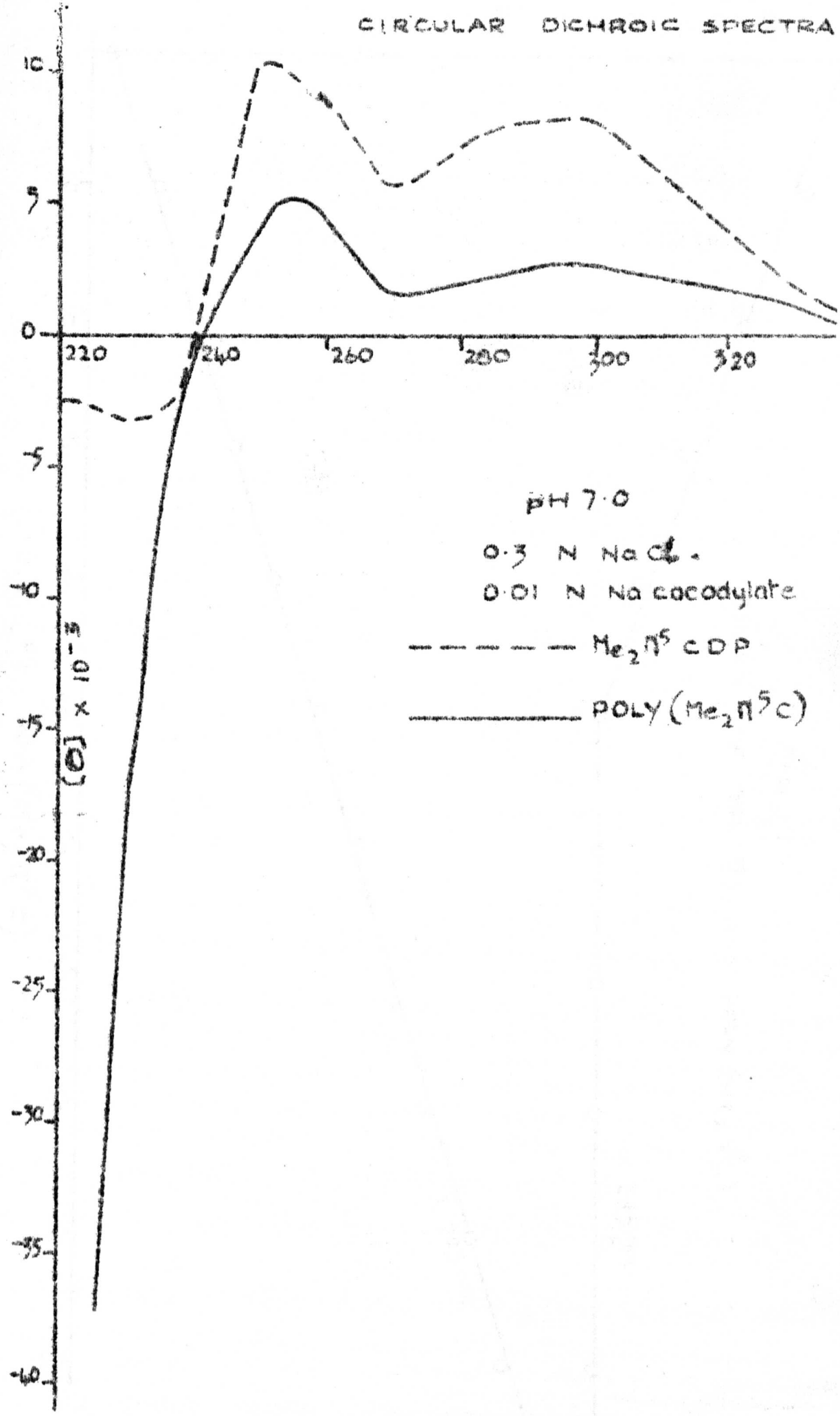


Fig 11

Mixing of poly (I) and poly (C)

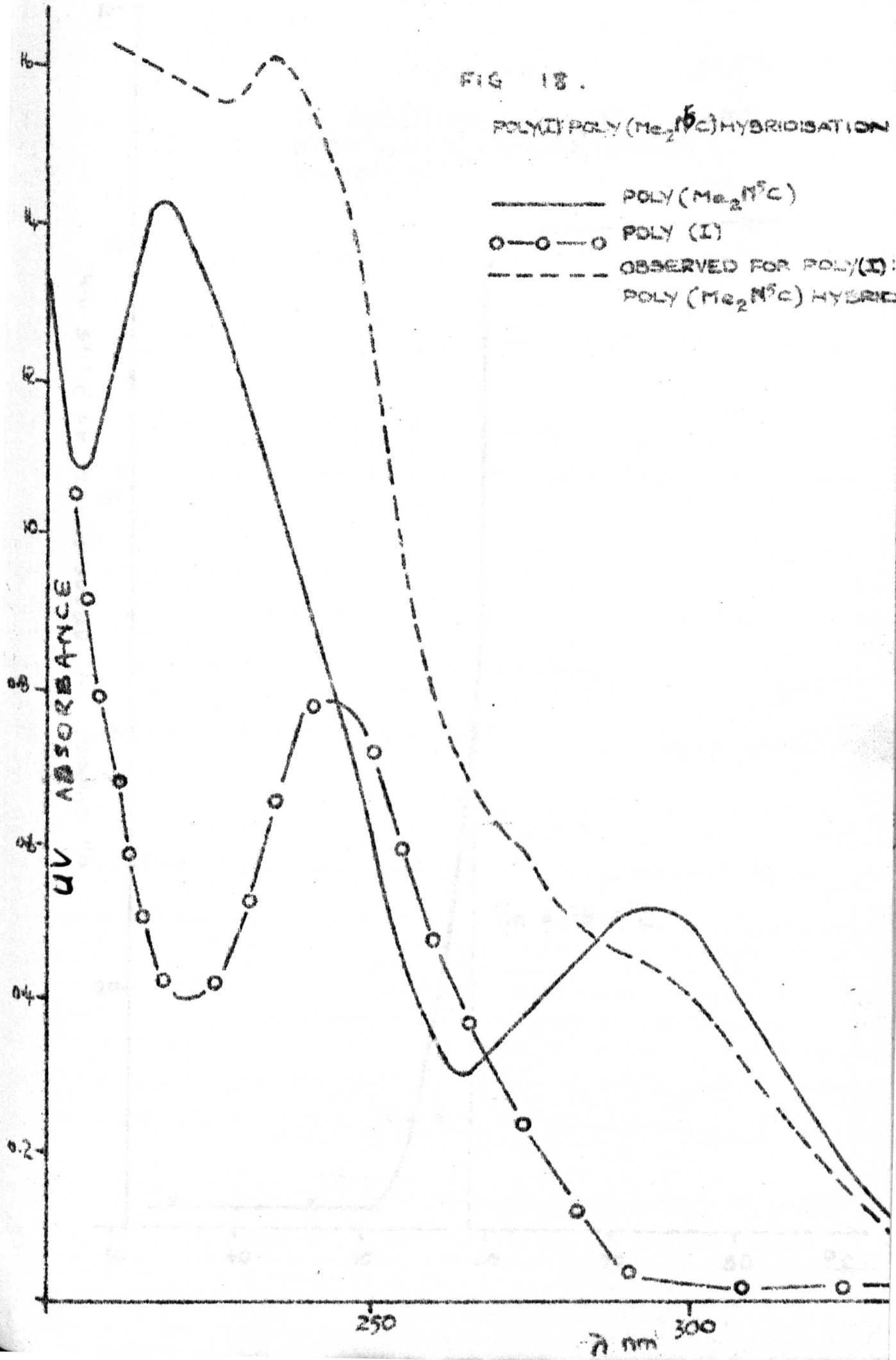


FIG 18.

POLY(I) POLY (Me_2N^5C) HYBRIDISATION

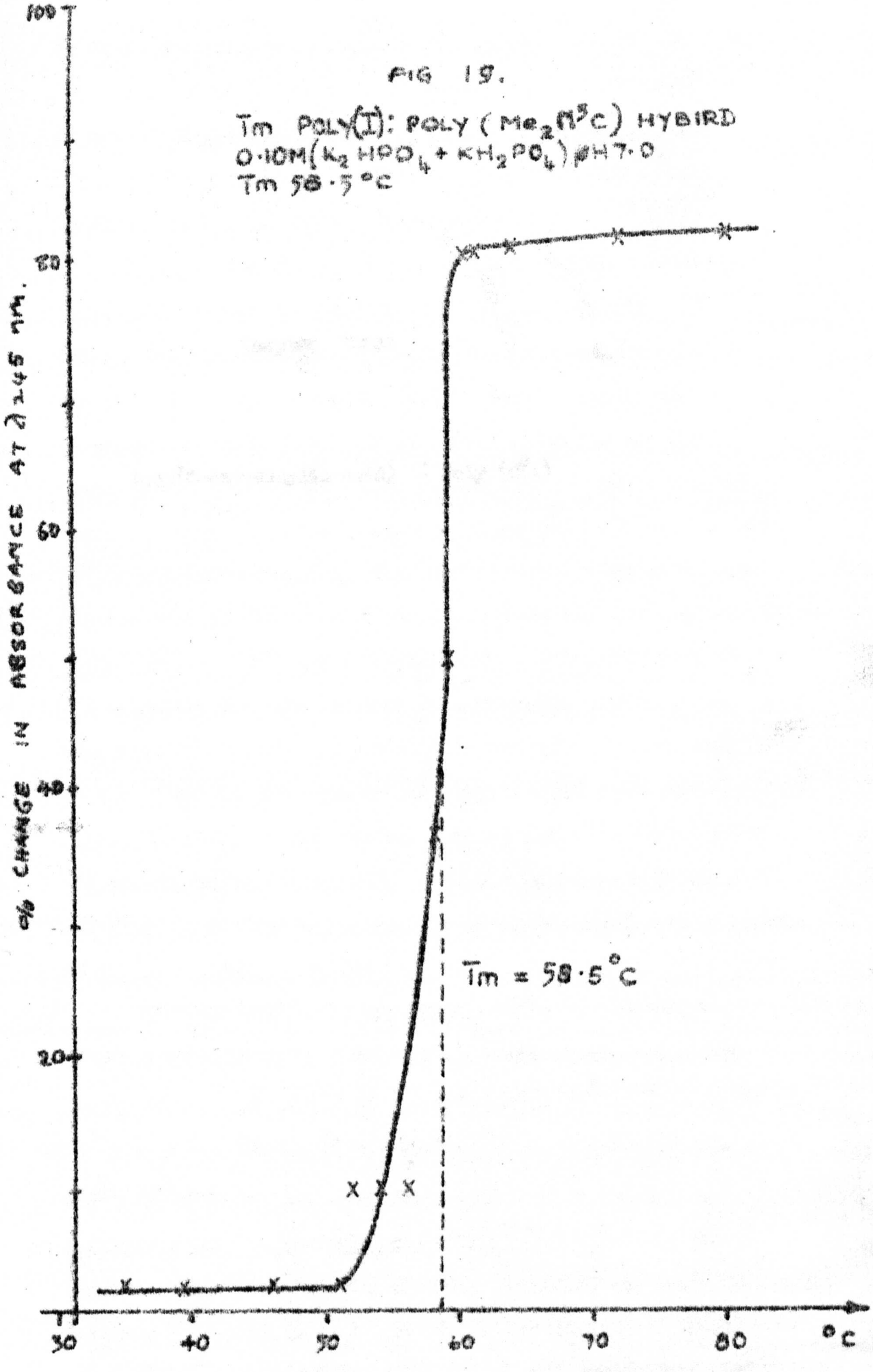
——— POLY (Me_2N^5C)

o—o—o POLY (I)

– – – – OBSERVED FOR POLY(I): POLY (Me_2N^5C) HYBRID

FIG 19.

Tm POLY(I): POLY (Me_2N^5C) HYBIRD
0·10M($K_2HPO_4 + KH_2PO_4$) @H 7·0
Tm 58·5 °C



Chapter Three

Poly(8-oxyadenylic Acid) : Poly (o^8A) .

8-Bromoadenosine 5'-monophosphate (br^8AMP):- This was prepared by a modified method of Munayama et al[261] for the preparation of 8-bromo 3',5'-cyclic adenylic acid. 5' AMP (1.32 g) was dissolved in water (5 ml) adding a few drops of dilute sodium hydroxide to aid solution. Sodium acetate (1M), (pH 3.7), (25 ml), was added and the pH kept below 4 by adding drops of dilute HCl. A further quantity of sodium acetate (1M), (pH 3.9), (30 ml) containing bromine (0.5 ml) was added and the solution was stirred at room temperature for 20 hours. Cyclohexene (2 drops) was added to remove free bromine and the remaining yellow colour was discharged by the addition of sodium sulphite (1M), (10 ml)[262]. The solution was evaporated to dryness to give br^8AMP and sodium acetate. This crude product was always used for the next stage without further purification. For characterisation purposes br^8AMP was crystallised in dilute HCl pH 3 at 4°C overnight when the crystals were filtered. The filtrate was evaporated to dryness and passed down activated charcoal column[263] (1.5 x 10 cm) to give more br^8AMP with a total yield of 0.9 g, (65%). UV pH 2, λ max 267 nm ε 14,000, pH 7, λ max 205 nm ε 14,500, pH 11, λ max 273, ε 14,500; compared with the UV of AMP pH 2, λ max 257 nm, ε 15,000; pH 7, λ max 259 nm, ε 15,400; pH 11, λ max 265, ε 15,400.

'HNMR (60 MHz); τ 2.60, (1H s), (H^2); τ 4.00 (1H d), (J = 7.0 Hz), ($H^{1'}$), τ 5.80, (5H m), sugar protons; compared to the NMR spectra of AMP of τ 1.50, (1H s), (H^2); τ 1.85, (1H s), (H^8); τ 4.00, (1H d), (J = 7.0 Hz), ($H^{1'}$); τ 5.80, (5H m), sugar protons.

8-Oxyadenosine 5'-monophosphate (o^8AMP)[1261]. Br^8AMP (1 g) and sodium acetate (2 g) (or 1 g if the crude br^8AMP was used without removing the sodium acetate used in the bromination reaction) were suspended in acetic anhydride (10 ml)/glacial acetic acid (100 ml)

and heated under reflux at 100-120°C for 4 hours. The resulting dark coloured solution was evaporated to dryness and the residue was dissolved in ethanol (10 ml). Diethyl ether (30 ml) was added to precipitate the 8-acetoxyadenosine 5'-monophosphate. The precipitate was filtered to give brown crystals, this crude product was not further purified before the next stage. It had UV spectra pH 2 λ max 293.5 nm, ε 12,000, pH 7 287.5 nm, ε 12,800; pH 12 λ max 294.5 nm, ε 12,600. This crude material was dissolved in methanol (100 ml) containing ammonium hydroxide (20 ml). The solution was heated under reflux at 55°C overnight to deacetylate the 8-position yielding free 8-oxyadenosine 5'-monophosphate. The solution was evaporated to dryness and the residue was dissolved in ethanol (10 ml). Diethyl ether (30 ml) was added to precipitate O^8AMP. The precipitate was filtered and purified on a column (1 cm x 20 cm) of carboxymethyl cellulose (H^+) eluted with water, to give the free acid of O^8AMP, yield 0.5 g, (46%). UV pH 2 λ max 263.5 nm, ε 8,800, 286 nm ε 7,500; pH 7 λ max 270 nm, 10,600, 255 nm, sh, ε 6,500, pH 11 λ max 281 nm ε 12,300, compared to the literature value for O^8, 3',5'-cyclic AMP[245] of UV pH 1, λ max 265.4 nm, sh 286.5 nm pH 7, λ max 270 nm, sh 255 nm; pH 12, λ max 281 nm.

^{1}HNMR (60 MHz), τ 2.60, (1H s), (H^2); τ 4.00 (1H d), (J = 7.0 Hz), ($H^{1'}$); τ 5.80, (5H m), sugar protons.

Dephosphorylation as described for Me_2N^5CMP above showed homogeneity with Rf on paper (system H) of 0.45 compared with the Rf of 0.37 for adenosine, and an Rf on TLC (system A) of 0.56 compared with an Rf of 0.42 for adenosine.

<u>8-Oxyadenosine 5'-diphosphate : O^8ADP:-</u> O^8AMP (100 mg) was converted into trisodium O^8ADP as described by Moffat and Khorana[257],

to yield 44 mg, (38%), chromatographically pure and electrophoretically homogeneous Rf on system D was 0.52 as compared to the Rf of 0.76 for 0^8AMP and electrophoretic mobility 0.64 as compared to the electrophoretic mobility of 0.40 for 0^8AMP. UV pH 2 λ max 263 nm, ε 9,500, 285 nm ε 8,500; pH 7, λ max 270 nm, ε 11,000, sh 255 nm, ε 7,000; pH 12, λ max 281 nm ε 12,500.

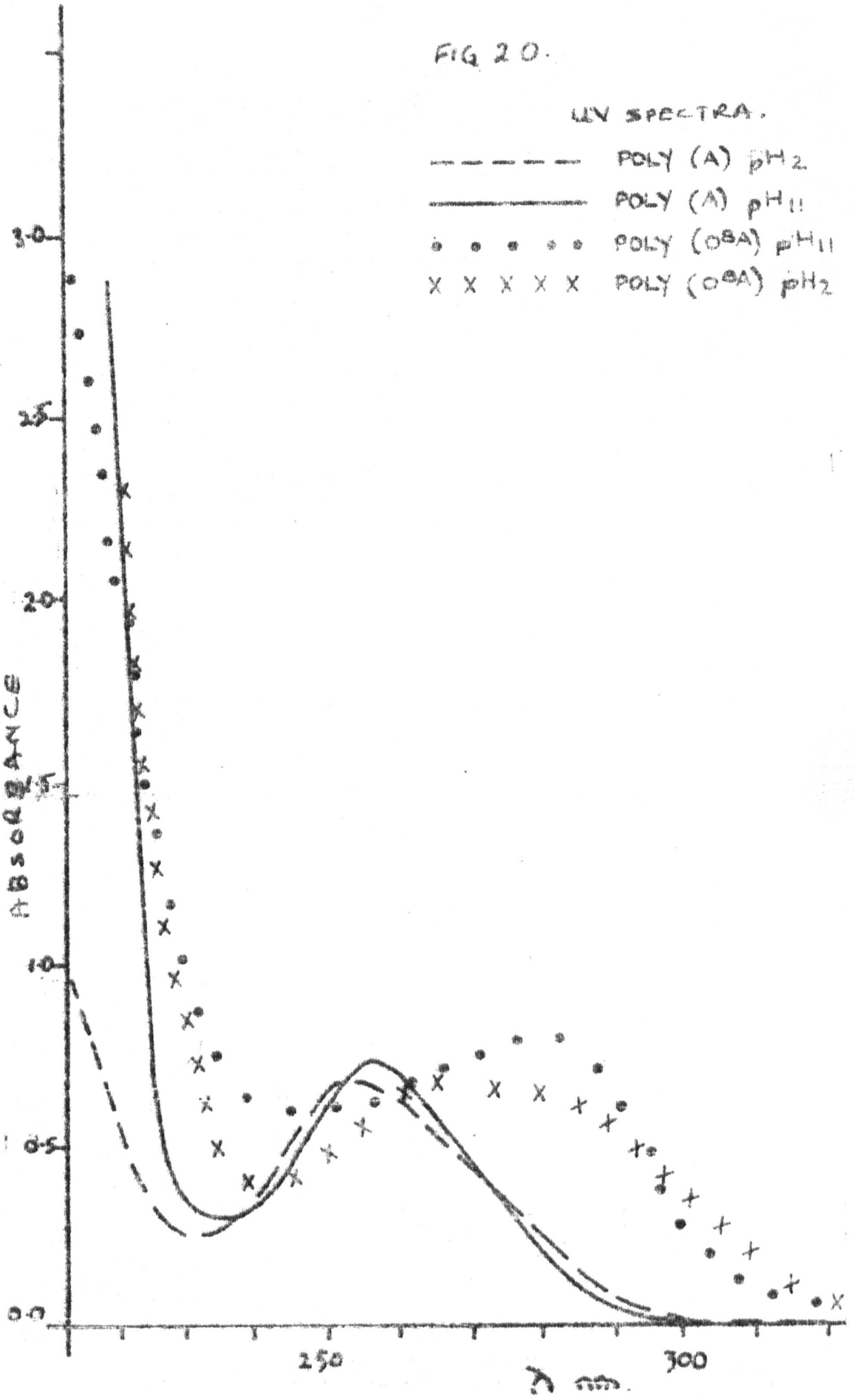
^{1}HNMR (60 MHz); τ 2.60 (1H s), (H^2); τ 4.10 (1H d), (J = 7.0 Hz), ($H^{1'}$); τ 5.80, (5H m), sugar protons.

Poly (8-oxyadenylic acid) : Poly (0^8A) :- The polymerisation of 0^8ADP was followed by inorganic phosphate release[258] at different ionic strengths, pH and temperature, but the reaction was too slow to give data from which meaningful Vmax and Km could be calculated. In a typical case the reaction medium which contained 0^8ADP (50 mg), PNPase (2.5 mg), albumin (1.0 mg) was incubated in a buffer (5 ml) of 0.2M Tris base, 6.7 mM EDTA Na, 13.3 mM $MgCl_2.6H_2O$, sodium azide (0.02 mg) pH 9.0 at 45°C for 22 hours. After deproteinisation and desalting as described for the preparation of poly($f1^5$C) a yield of 4.6 mg, (9%) was obtained as determined by UV absorbance.

Characterisation of poly(0^8A):- Poly(0^8A) ran as a single compound on gel electrophoresis and had an $S_{20,w}$ of 7.2 by ultracentrifugation in an isokinetic gradient of sucrose containing sodium acetate (pH 7.0). UV in 0.01M sodium acetate pH 2 λ max 265 nm, ε 8,000, sh 285 nm, ε 7,000; pH 7, λ max 270 nm, ε 10,000, sh 255 nm, ε 6,000; pH 12, λ max 281 nm, ε 11,000 (fig. 20).

Sodium hydroxide digest:- Heating a solution of poly(0^8A) (0.2 μM) in 0.1N sodium hydroxide for 15 minutes at 90°C caused only a hyperchromic rise of 1.3% at λ 281 nm; under similar

FIG 20.


UV SPECTRA.
POLY (A) pH 2
POLY (A) pH 11
POLY (OBA) pH 11
POLY (OBA) pH 2
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ABSORBANCE
250
300
λ nm.

conditions poly(A) gave 36% hyperchromic rise (fig. 21).

PRNase digest:- Poly(O^8A), (0.2 μM) in 0.1M sodium acetate (pH 7.0), (2 ml), was treated with PRNase (0.5 μg). The half life of the hydrolysis was 54 sec with a hyperchromic rise at 270 nm of 1%.

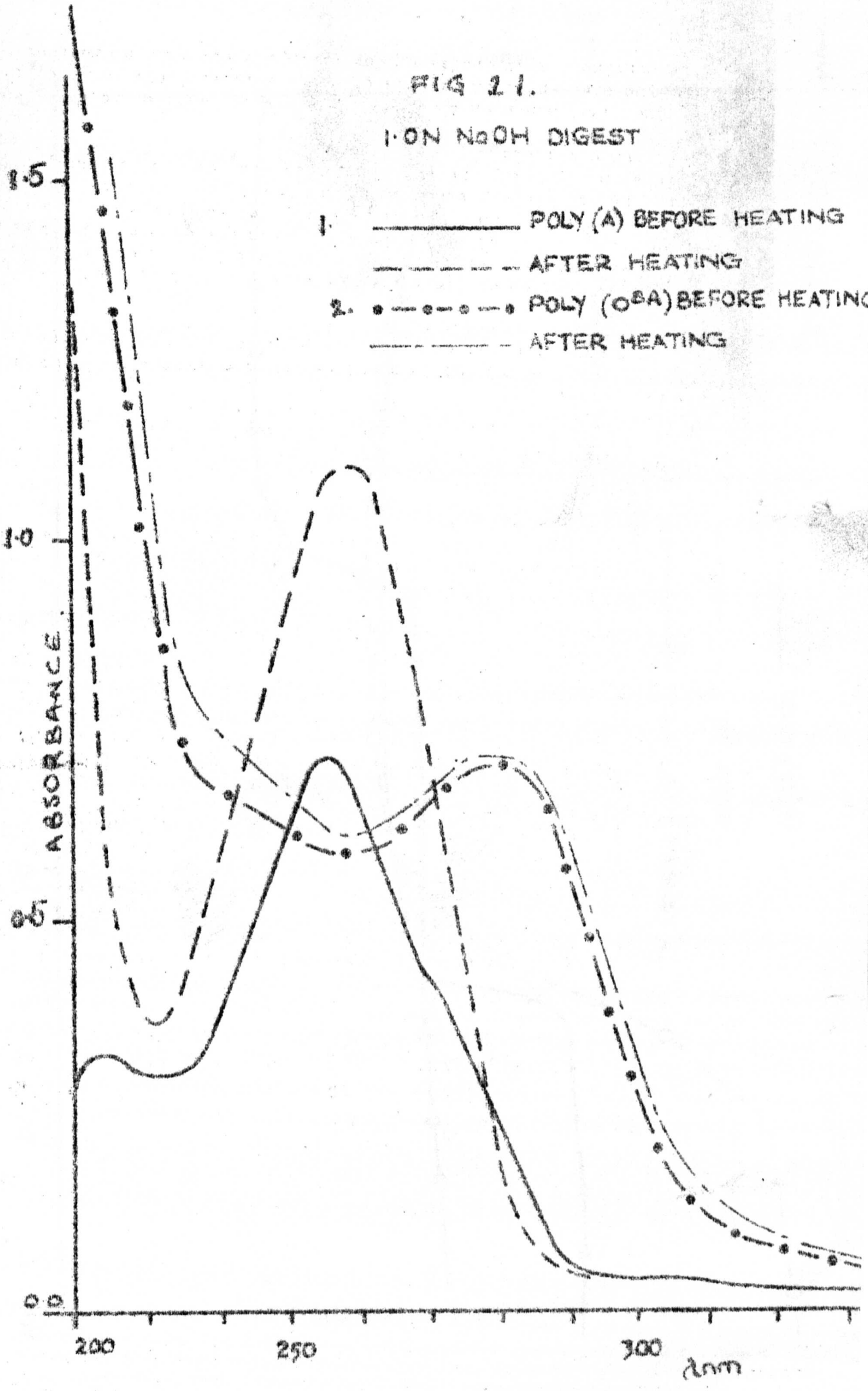
pH Titration:- Titration of poly(O^8A) at 270 nm as described for poly($f1^5$C) did not exhibit any sharp fall as for poly(A), but a gradual drop as for O^8ADP in the region of pH 5 - 6. There was however a fairly sharp drop in the region of pH 9 as compared with a gradual slope of O^8ADP in this region. Poly(A) under these conditions showed a 14% hypochromicity at pH 5.7 (see fig. 22).

Circular dichroic spectra:- Poly(O^8A) showed very low CD absorbance in 0.001N HCl with a positive absorbance between λ217 nm and 300 nm with λ max 258 nm and molar ellipticity of 5,000 and a negative absorbance between λ205 nm and λ 217 nm with λ min 210 nm and molar ellipticity of -2,500. At pH 7.0, λmax was 220 nm and sh 270 nm with molar ellipticities of 15,000 and 7,000 respectively and λ min 205 nm with molar ellipticity of -30,000. In 0.001N sodium hydroxide λmax was 248 nm and λ min 195 nm with molar ellipticities of 17,000 and -16,000 respectively (fig. 23). O^8ADP in 0.001N HCl had λ max 250 nm and λ min 218 nm with molar ellipticities of 5,000 and -5,000 respectively. At pH 7.0 λmax 242 nm λmin below 200 nm with molar ellipticities of 7,000 and below -6,000 respectively. In 0.001N sodium hydroxide λ max 240 nm and λ min 208 nm with molar ellipticities of 5,000 and -8,000 respectively (fig. 24).

Preparation of poly(O^8A).poly(U)$_2$:- When equimolar solutions of poly(O^8A) and poly(U) were studied by the method of continuous

FIG 21.

1·0N NaOH DIGEST



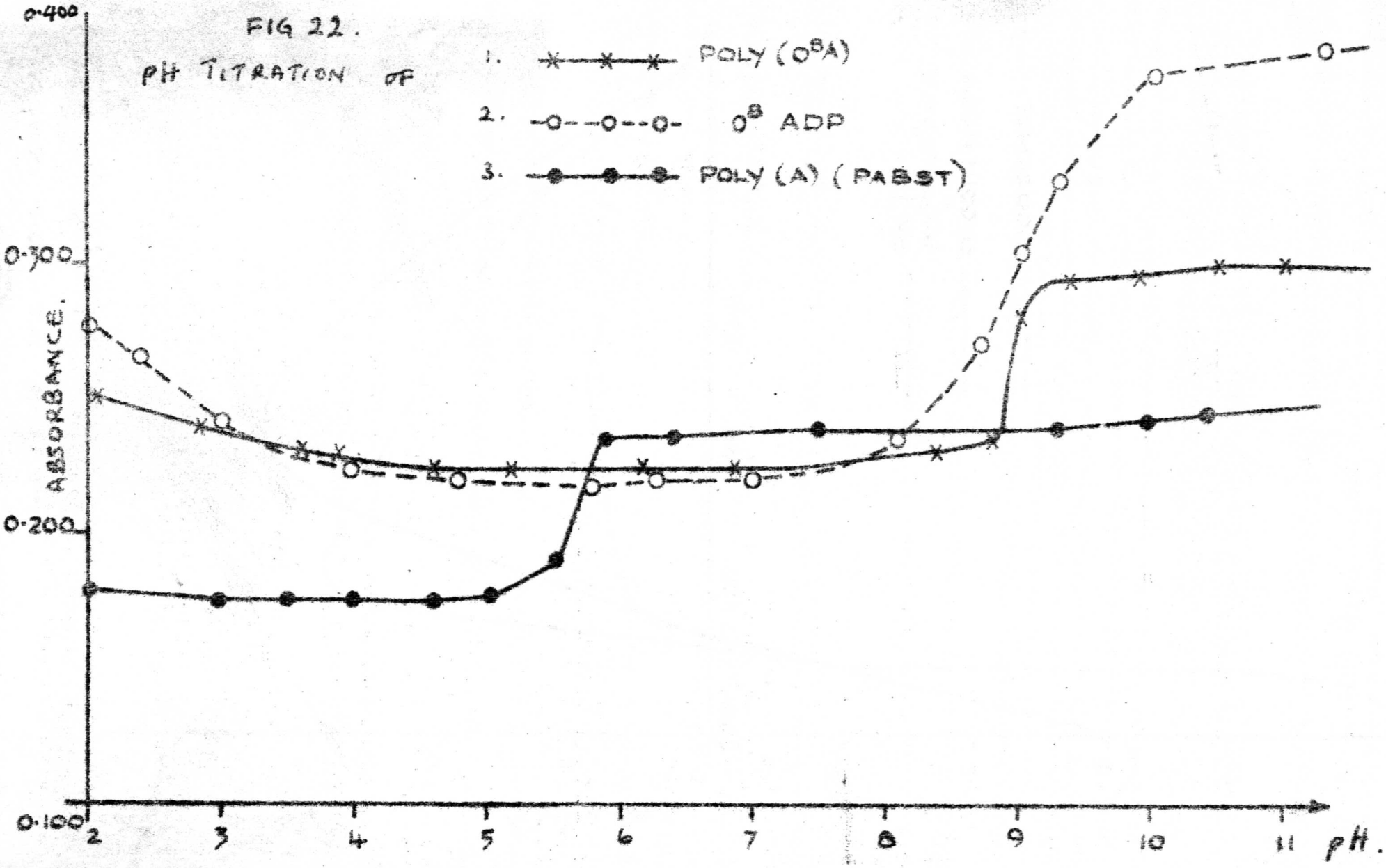


FIG 22.
PH TITRATION OF
1. POLY (O^BA)
2. O^B ADP
3. POLY (A) (PABST)
0·400
0·300
0·200
0·100
ABSORBANCE.
2
3
4
5
6
7
8
9
10
11
pH.

FIG 23.

CIRCULAR DICHROIC SPECTRA OF POLY ($O^{8}A$)

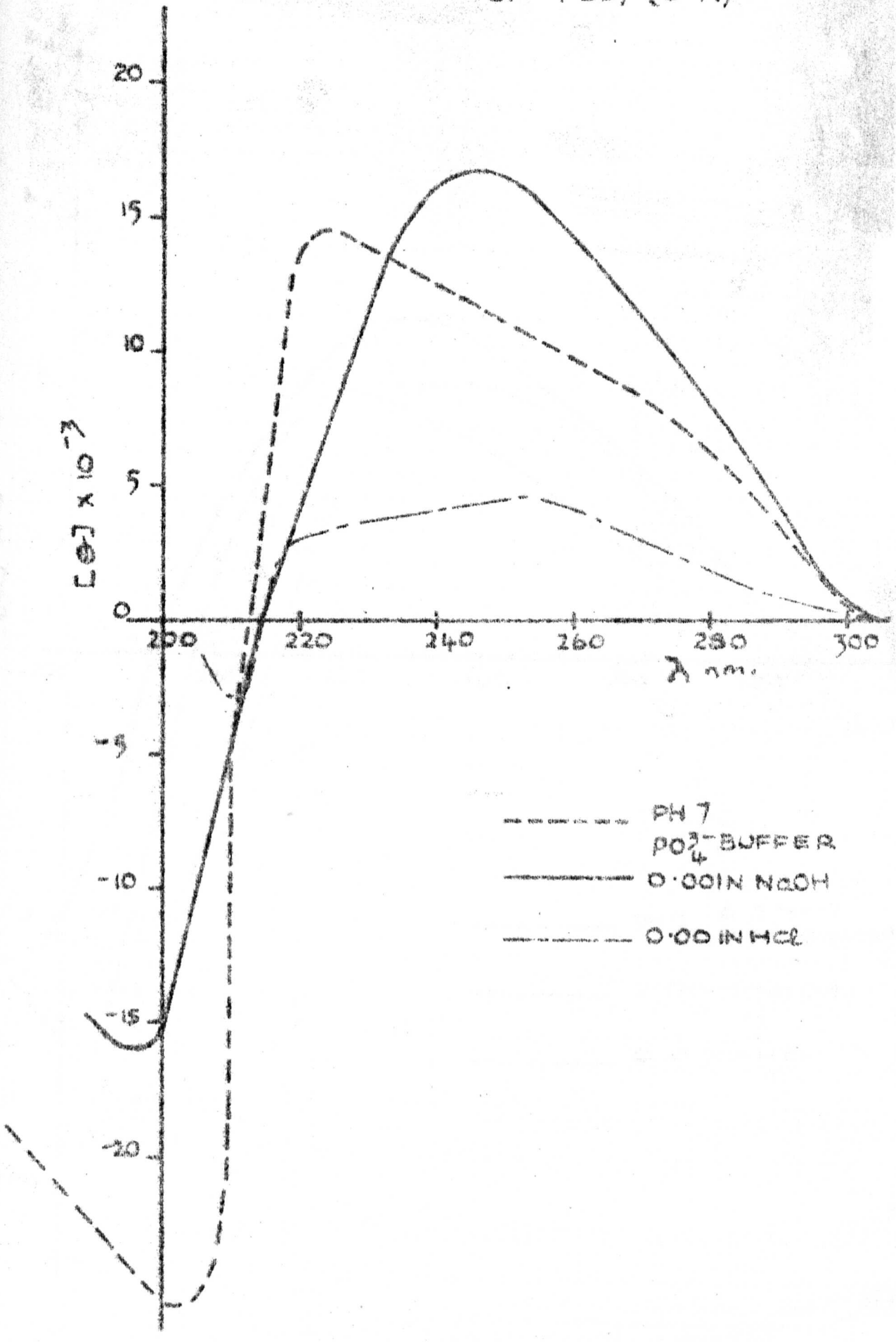
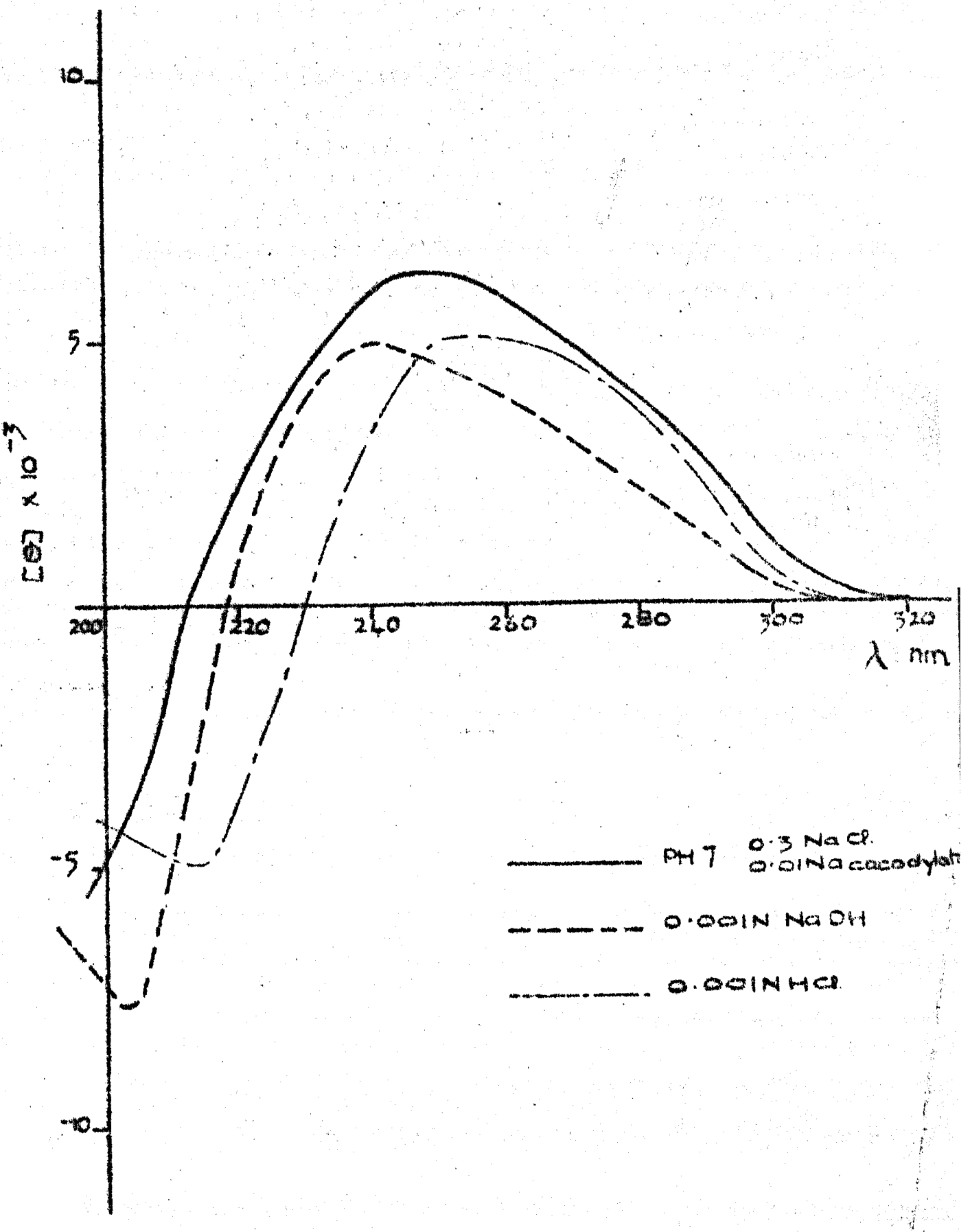


FIG 24.

CIRCULAR DICHROIC SPECTRA OF O^B ADP



variations the results were not good enough for a reasonable plot as the spectra did not intercept (fig. 25). At 1:2 molar solutions however, a plot was obtained with a deflection at a 50:50 mixture of these 1:2 molar solutions, indicating a 1:2 hybrid formation of poly(0^8A) $S_{20,W}$ 7.2 and poly(U) $S_{20,W}$ 7.5 in (0.01M K_2HPO_4 + KH_2PO_4), pH 7.0 at 37°C (fig. 26). The Tm of the hybrid was found as described for poly(I).poly($f1^5$C), in 0.01N and 0.1N sodium chloride to be 60°C and 61°C respectively with a hyperchromicity of 4% (fig. 27). The hybrid was not sent for interferon assay as it was a triple stranded hybrid.

FIG 25.

HYBRIDISATION OF POLY (O^{6}A) WITH POLY (U)

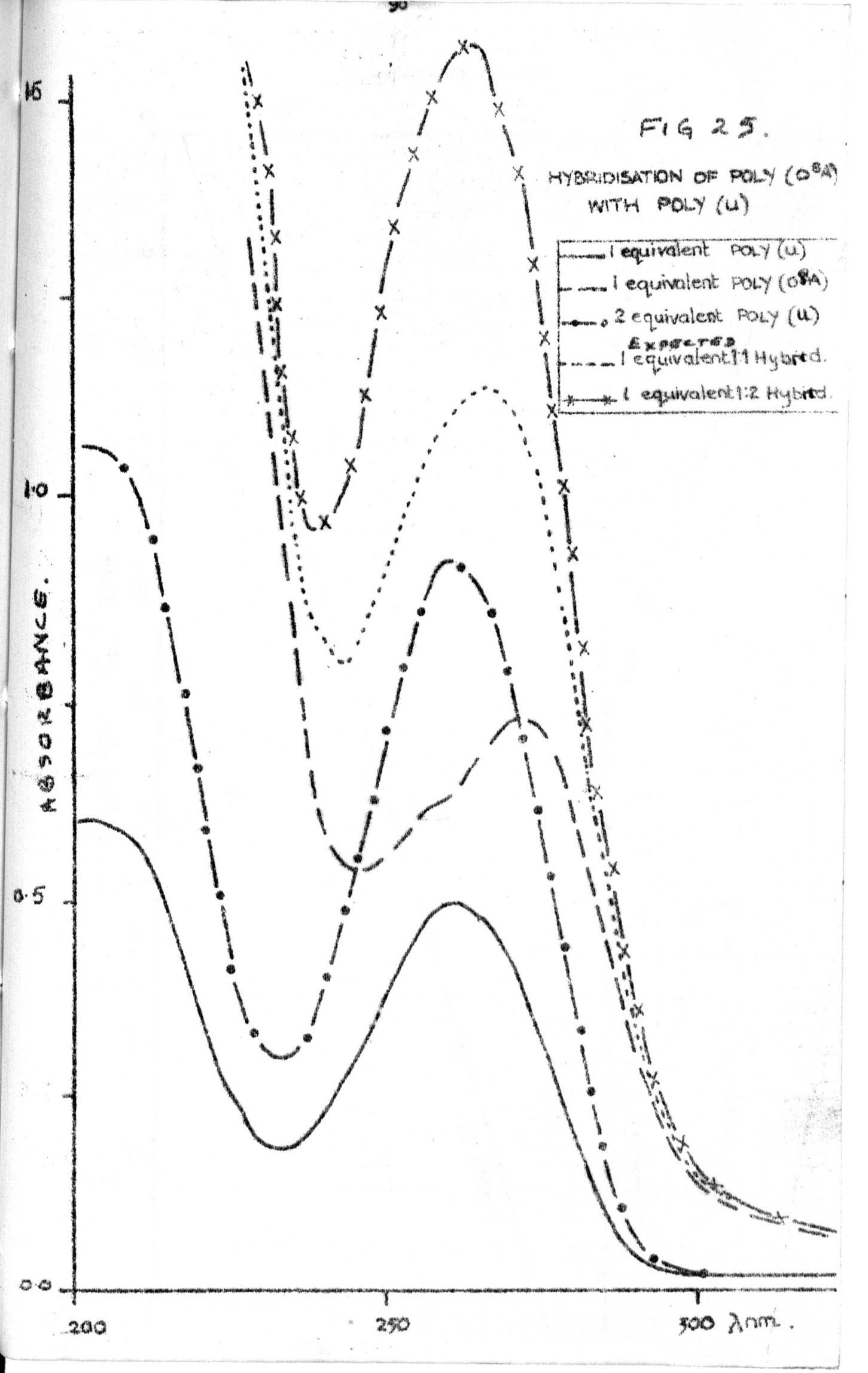


FIG 26.

HYBRIDISATION OF POLY(OBA) AND POLY $(U)_2$.

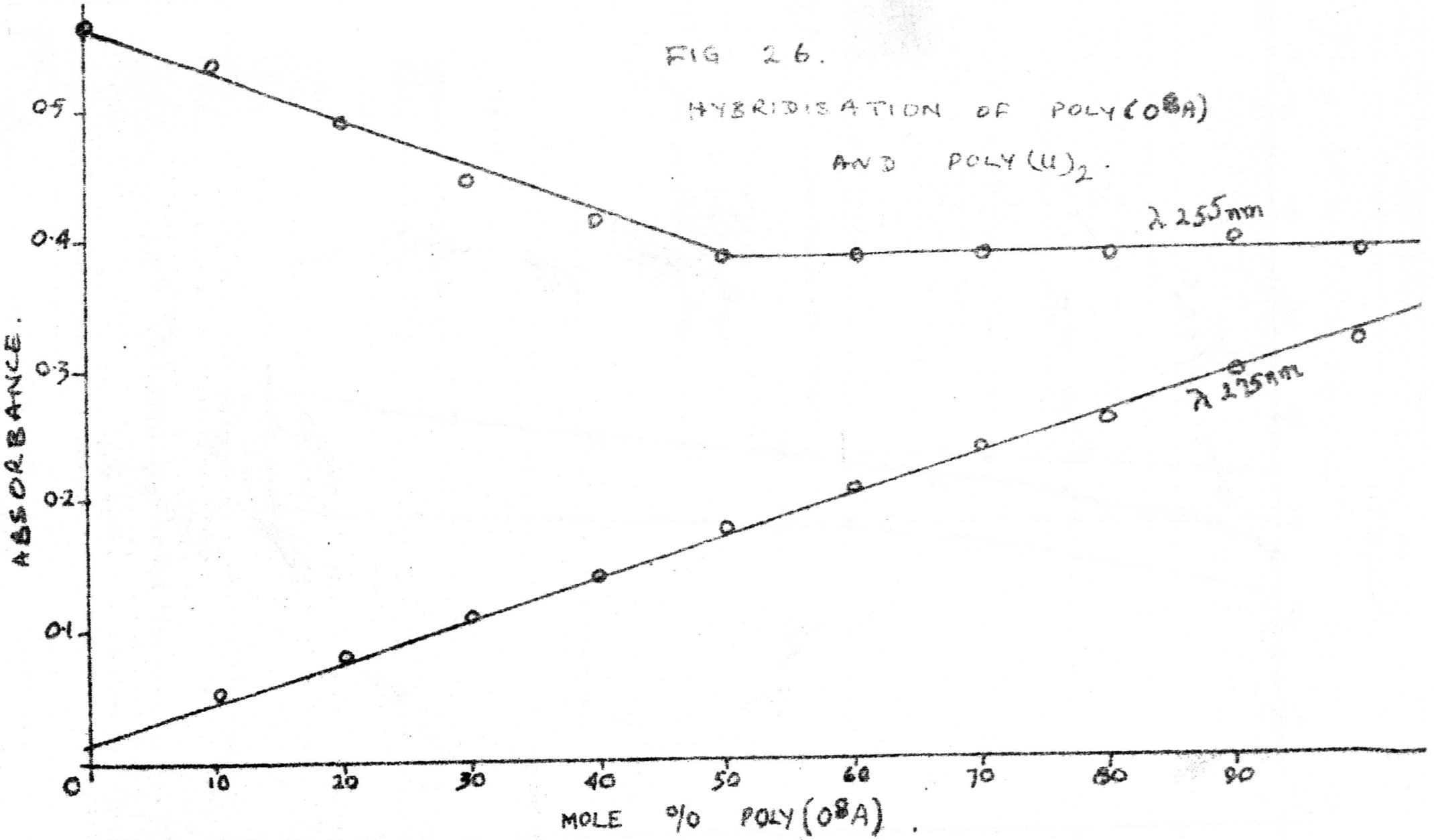
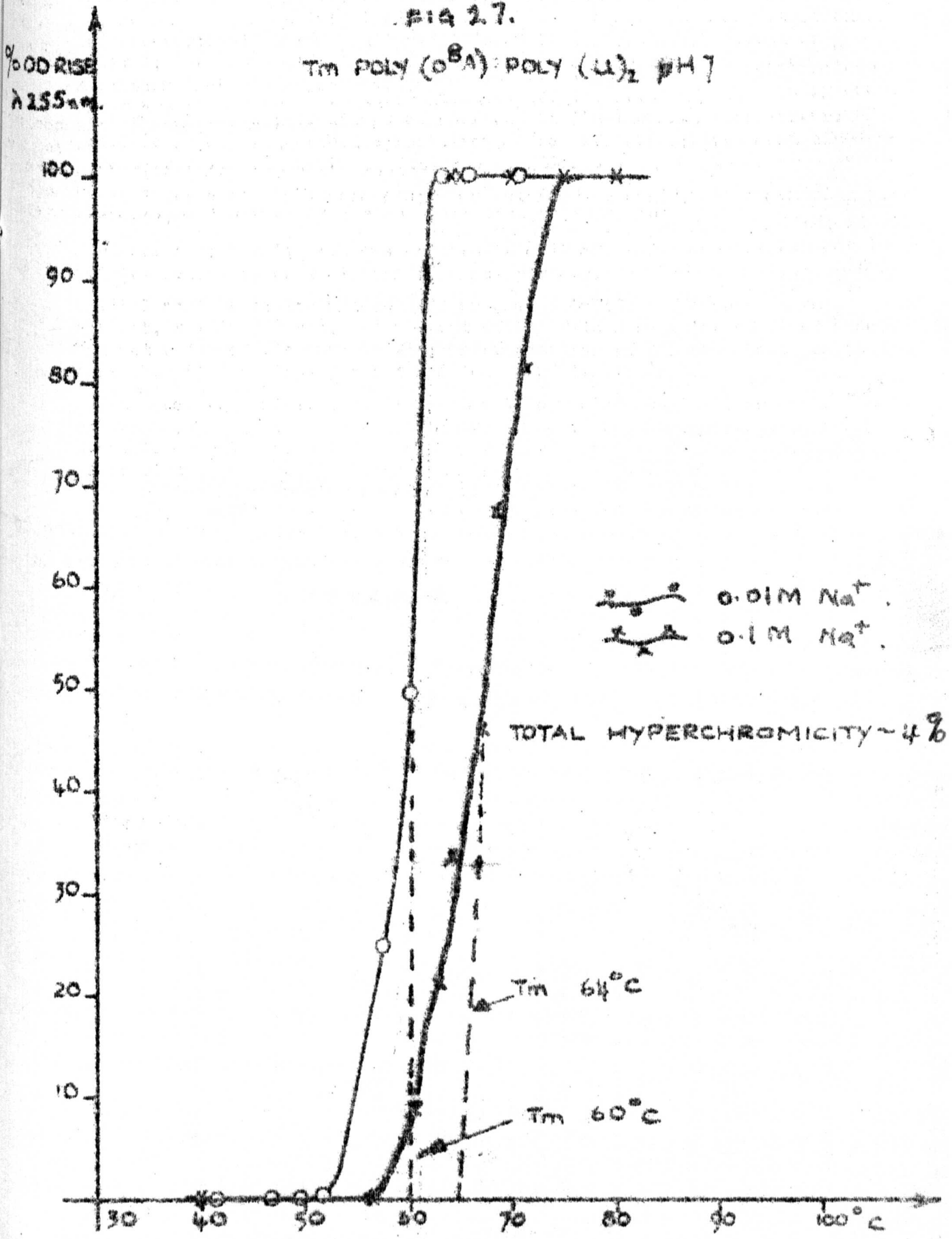


FIG 2.7.

Tm POLY ($o^{8}A$) : POLY $(U)_2$ [pH 7]



A P P E N D I X

APPENDIX 1

In earlier work in this laboratory[69] the rates of hydrolysis of 5-substituted uridine and cytidine 2',3'-cyclic phosphate were compared with those of the unsubstituted analogues. In addition, the rates of hydrolysis of poly (5-substituted cytidylic acids) were also determined. While that work was in progress the results of a similar study was published by other authors[73].

Recorded here is further work done on the hydrolysis of 2',3'-cyclic monophosphate nucleotide. The results as shown agree with the earlier findings[69,73] that there is no significant difference in the rate of hydrolysis of the 5-substituted and the unsubstituted nucleotide at the 2',3'-cyclic phosphate level.

5-Hydroxyuridine 5'-monophosphate[264]. UMP (1 g) was dissolved in water (20 ml) and bromine (0.5 ml) was added after which nitrogen was bubbled through the solution until it became colourless. Pyridine (5 ml) was added to the solution which was refluxed on boiling water bath for 15 hours when the yellow solution was evaporated to dryness and the residue crystallised in hot ethanol to yield 600 mg, (55%) needle-like crystals which melted at 242-244°C. Analysis calculated for $C_9H_{12}O_7N_2P$; C,41.56; H,4.61; N,10.7%; found C,41.36; H,4.92; N,10.30%. UV pH 1 λmax 278 nm, ε 7,500; pH 7 λmax 282 nm, ε 7,000; pH 12 λmax 302 nm, ε 6,600, chromatographically pure, with Rf on paper (system D) of 0.54 compared to Rf 0.42 for uridine 5'-monophosphate.
IR:- Broad keto band 1640 - 1610 cm^{-1} compared to the uridine 5'-monophosphate band at 1660 cm^{-1} and 5-bromo uridine 5'-monophosphate at 1660 and 1625 cm^{-1}.

^{1}HNMR (60 MHz):- τ 2.30 (1H s), (H^6); τ 4.25, (1H d), (J = 2.0 Hz), ($H^{1'}$) τ 5.5 - 6.0, (5 H m), sugar protons.

5-Hydroxyuridine 2',3'-cyclic monophosphate:-
5-Hydroxyuridine 2',(3')-monophosphate (500 mg)(mixed isomers) made as described for 5-hydroxyuridine 5'-monophosphate above, was converted to the sodium salt form by passing down Dowex 50 Na^+ column (1 cm x 30 cm) eluting with water, and eluants evaporated to dryness. The residue was dissolved in dry methanol (30 ml) and dicyclohexylcarbodiimide (2 g) was added after which the solution was stirred at room temperature for 24 hours and evaporated to dryness. The nucleotide was extracted with water (20 ml x 3) and the solution extracted with ether (10 ml x 3) before it was evaporated to dryness. The residue was crystallised to yield 250 mg, (54%), chromatographically pure with Rf on

paper (system D) 0.48 compared to Rf 0.40 of uridine 2',3'-cyclicmonophosphate, and broad band of the mixed isomers of uridine 2',(3')-monophosphate of Rf 0.34. UV pH 1 λmax 277 nm ε 7,000; pH 7 λmax 277 nm ε 6,700; pH 12 λmax 303 nm ε 6,200.

I.R.:- Broad keto band 1640 - 1610 cm^{-1}.

^{1}HNMR(60 MHz):- τ 2.30 (1H s), (H^6); τ 4.25, (1H d), (J = 2.0 Hz), ($H^{1'}$); τ 5.5 - 6.0, (5 H m), sugar protons.

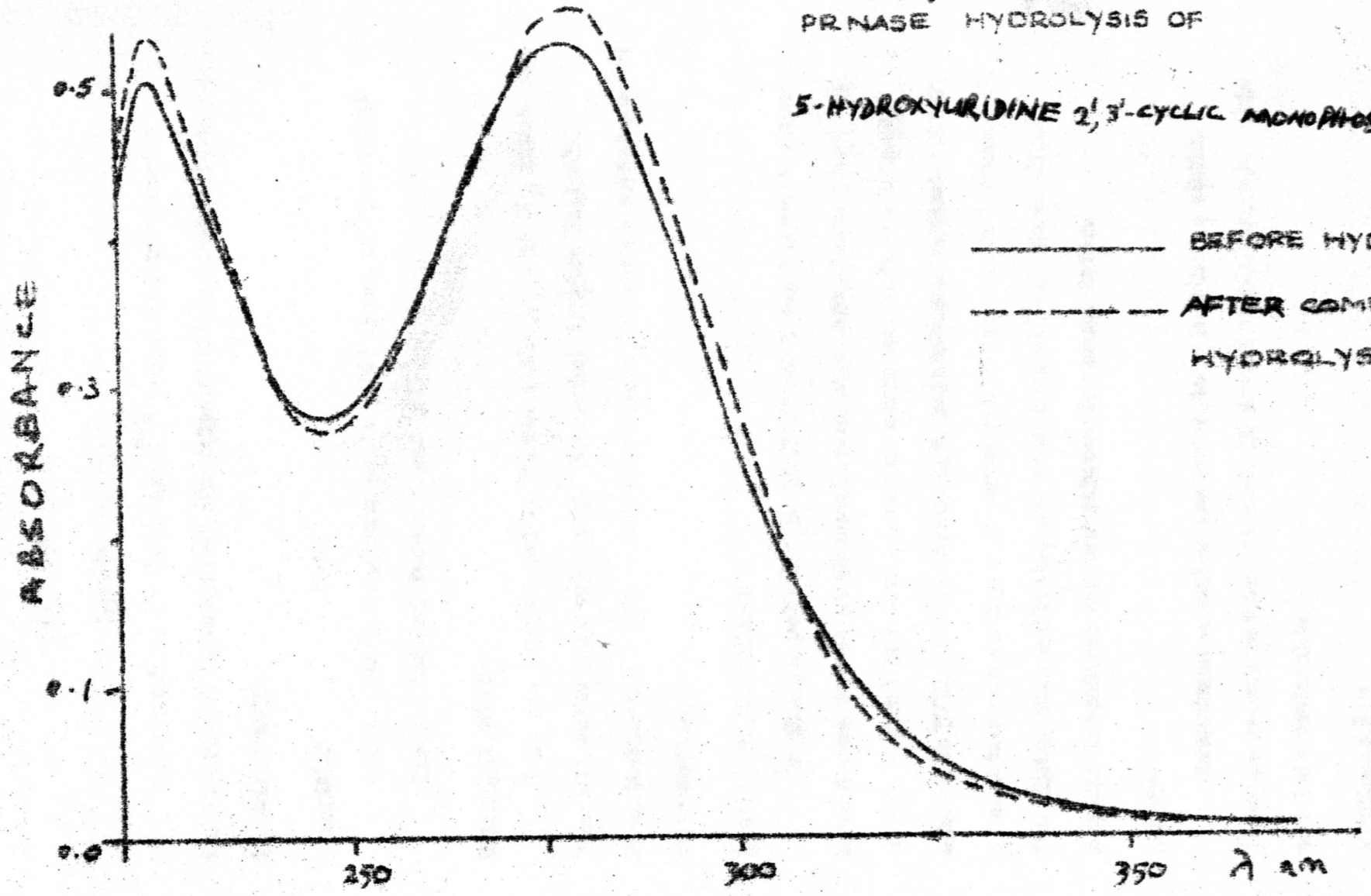
PRNase digest:- 5-hydroxyuridine 2',3'-cyclic monophosphate (0.2 μM) in 0.1M sodium acetate (pH 7.0), (2 ml) was treated with PRNase (1.0 μg). The half life of the hydrolysis was 4 hours which was twice as slow as the hydrolysis of uridine 2',3'-cyclic monophosphate of half life 2 hours. The product of the reaction 5-hydroxyuridine 3'-monophosphate had UV λ max pH 7.0 279 nm ε 7,000, (fig. 28).

	Uridine 2',3'-cyclic monophosphate	5-hydroxyuridine 2',3'-cyclic monophosphate
UV pH 7 λmax nm	259	277
	9,500	6,700
Vmax log $\frac{A_\infty - A_o}{A_\infty A_o}$ sec^{-1}	3,387	1,716
Km (μM)	10.5	5.0
$t_{\frac{1}{2}}$ (hours)	2	4

A_o and A_∞ are absorbance at time zero and absorbance at infinite time respectively.

FIG 28.
PRNASE HYDROLYSIS OF

5-HYDROXYURIDINE 2', 3'-CYCLIC MONOPHOSPHATE.



APPENDIX II

Interferon Assay

Reported here is Johnston's[214] techniques of assaying the interferon induction of the polynucleotide hybrids reported in this thesis.

Materials:

Cells: Human Embryonic Cells (HEC) known as Flow 1,000

Virus: Semliki Forest Virus (SFV).

Growth of cells:-

This was carried out in Unicam vials in a 5% CO_2 incubator. When all cells were confluent, the medium (Engles Minimal Essential Medium, MEM, + 2mM glutamine + 10% foetal bovine serum) was removed.

Interferon production:

In plastic petridishes of diameter 5 cm, polynucleotide hybrids were stored at 50 µg/ml in maintenance medium (same as growth medium) but with no bovine serum and left for 1 hour, always at 37°C, washed thrice with maintainance medium. 2 mls of maintainance medium + 2% serum was added and left overnight. The fluids were collected and stored at -20°C. These fluids contain interferon and are diluted for assay later.

Assay:-

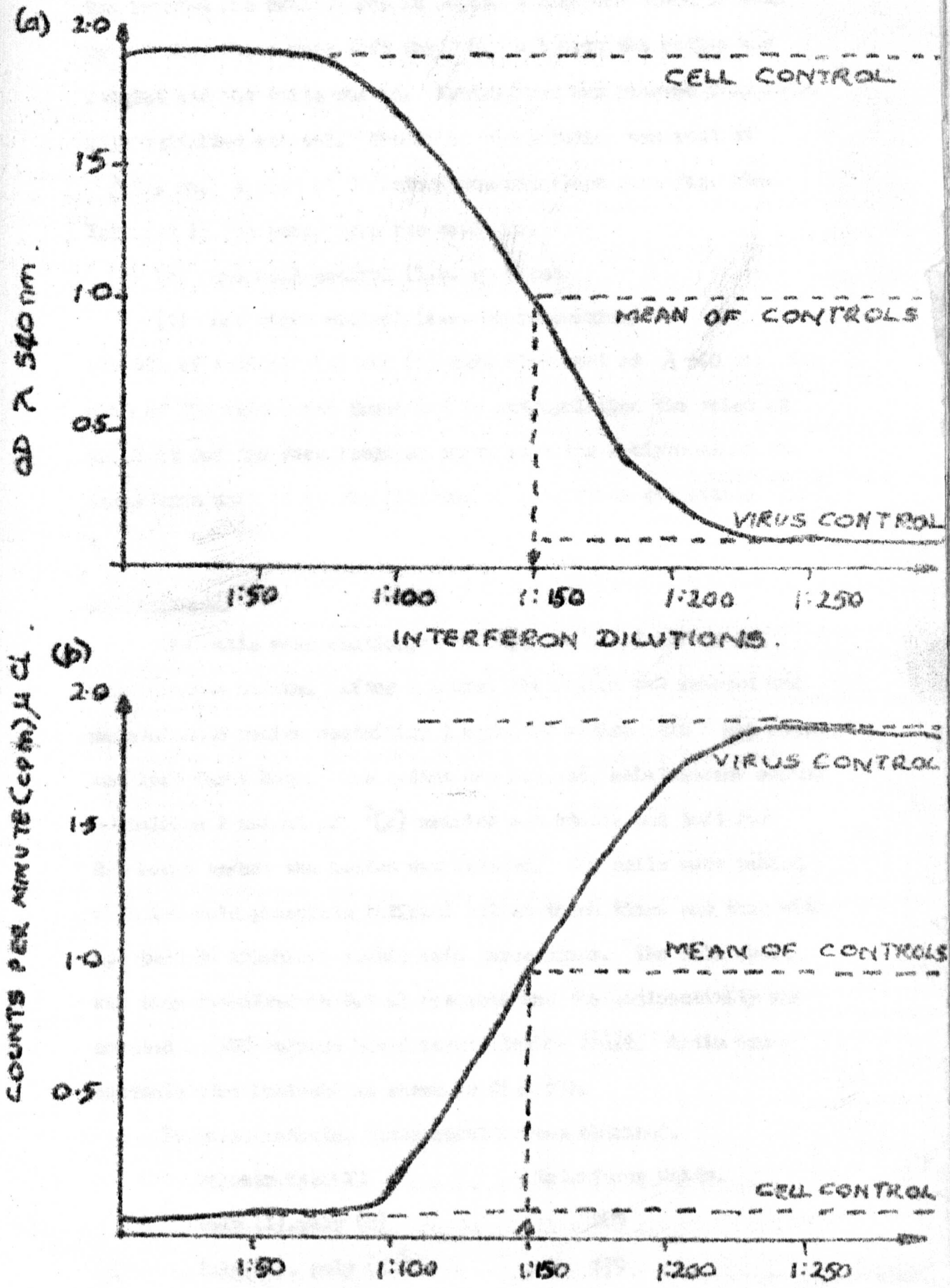
Interferon solution was added to maintainance medium (same as growth medium but with 2% foetal bovine serum). This was left overnight.

Procedure I:-

In a number of petridishes, dilutions of interferon were made as shown in fig. 29. The cells were challenged with 0.1 pfu/ml (plaque forming unit) and left for 48 hours. The supernatant

FIG 29.

DOSE RESPONSE CURVES



was removed and neutral red in Eagles saline was added to each petridish. These were left for 2 hours before the medium was removed and the cells washed. Neutral red was removed from cells with acidified ethanol. The OD of the solution was read at λ 540 nm. A plot of OD/interferon dilutions gave fig. 29a. Included in the assay were two controls.

(a) one cell control (i.e. no virus)

(b) one virus control (i.e. no interferon)

The ODs of controls (a) and (b) were also read at λ 540 nm. The mean of the values was found and by extrapolation the point at which it cut the dose response curve gave the reciprocal of the interferon unit (i.e. the dilution of interferon containing 1 unit).

Procedure II:

The cells were challenged with 10 pfu/ml of SFV in maintainance medium. After 2 hours, the medium was removed and maintainance medium containing 1 μg/ml of actinomycin D was added and left for 1 hour. The medium was removed, maintainance medium containing 2 μCi/ml of ^{3}(H) uridine was added, and left for 2.5 hours before the medium was removed. The cells were washed with ice cold phosphate buffered saline three times and then with ice cold 5% trichlore acetic acid three times. The cell sheet was then dissolved in 0.2 ml protosol and the radioactivity was counted in 10% toluene based scintillation fluid. Again two controls were included as shown in fig. 27b.

By these methods, these results were obtained.

Polymer Hybrid.	Interferon Units.
Poly (I).poly (C)	200
Poly (I). poly ($f1^{5}C$)	150

Preliminary tests on poly (I).poly ($Me_2n^{5}C$) by Dr. T. Cartwright, Searle Laboratories, High Wycombe, showed some interferon induction.

Appendix 3.

Here are photocopies of some of the papers published from works reported in this thesis.

September 1973
Memo 1-906

ANTIVIRAL ACTIVITY OF POLY(5-FLUORORIBOCYTIDYLIC ACID): POLY (RIBOINOSINIC ACID)

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Poly rI will form hybrids with poly(br^5-, cl^5- or f^5rC) which have higher T_ms than poly rI.poly rC and which are inducers of interferon. Poly f^5rC can be prepared by the polymerisation of f^5CDP using polynucleotide phosphorylase and is more resistant to hydrolysis by pancreatic RNase than poly rC. Poly f^5rC($s_{20,w}$ 7.2) was annealed with poly rI($s_{20,w}$ 6.5) in 0.1 M phosphate buffer to give a 1:1 hybrid with a T_m in 0.1 M salt of 67°.

The hybrid poly rI.poly f^5rC in PBS at a concentration of 50 µg/ml was compared with poly rI.poly rC as an interferon inducer in human embryo fibroblast cells. The assay which also used human embryo fibroblast cells was based on the inhibition of Semliki Forest virus nucleic acid synthesis. Poly rI.poly rC gave 200 units and poly rI.poly f^5rC, 150 units of interferon under these conditions.

Tetrahedron Letters No. 51, pp 5077 - 5080, 1973. Pergamon Press. Printed in Great Britain.

*POLY (5-DIMETHYLAMINOCYTIDYLIC ACID)

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(Received in UK 27 September 1973; accepted for publication 8 November 1973)

We are investigating the biological properties of polycytidylic acids which are substituted in the pyrimidine ring (e.g. poly(cl^5C)[1] and poly(ho^5C)[2]. These polynucleotides can be prepared by polymerising the corresponding nucleoside diphosphates with polynucleotide phosphorylase. In this paper we report the synthesis of 5-dimethylaminocytidine 5'-diphosphate (m_2n^5CDP) and its enzymic polymerisation to poly(5-dimethylaminocytidylic acid) [poly(m_2n^5C)].

5-Dimethylaminocytidine 5'-monophosphate - 5-Bromocytidine 5'-phosphoric acid[3] (1 g) was brought to pH 7 with tetrabutylammonium hydroxide, and the solution evaporated in vacuo, the last traces of water being removed at 0.1 mmHg. The gummy residue, dissolved in dry dimethylformamide (10 ml), was cooled to 0^o and dimethylamine (3 ml) was added. This reaction was kept at 60^o under a reflux condenser cooled with solid carbon dioxide/acetone for 8 hours, further portions of dimethylamine (3 ml) being added every 2 hours. The reaction vessel was then connected to a water-cooled condenser and left at 60^o overnight. Water (50 ml) was then added and hydrochloric acid to bring the pH of the solution to 2. The solution was then evaporated to dryness in vacuo, the residue dissolved in a little water and applied to a Dowex 50 column (H^+ form, 100-200 mesh, 2 x 36 cm). Elution with water gave a little ho^5CMP, followed by unreacted br^5CMP and finally m_2n^5CMP, evaporation of the last fraction gave 5-dimethylaminocytidine 5'-monophosphoric acid (472 mg, 40%) Found C, 34.34; H, 5.60; N, 14.41; P, 8.20% Calc. for $C_{11}H_{19}N_4O_8P.H_2O$ C, 34.38; H, 5.50; N, 14.57; P, 8.06%. Ultraviolet spectrum

pH 1 λ_{max} 312 (ε4,800), 218 nm (ε9,000); pH 7 λ_{max} 294 (ε6,000), 224 nm (ε17,000); pH 12 λ_{max} 294 (ε7,000), 224 nm (ε20,000). The pKa of (m_2n^5CDP) at 21° measured spectrophotometrically was 4.14 ± 0.07.

Dephosphorylation of m_2n^5CMP - This was carried out as previously described[4] to give 5-dimethylaminocytidine which ran as a single spot on paper and silica thin layer chromatograms.

5-Dimethylaminocytidine 5'-Diphosphate was prepared in 60% yield from m_2n^5CMP by the phosphoromorpholidate method[5] and was isolated as the trisodium salt. Found C, 26.47; H, 4.46; N, 10.14% Calc. for $C_{11}H_{17}N_4O_{11}P_2Na_3$ C, 25.79; H, 3.34; N, 10.93%. Ultraviolet spectrum, pH 1 λ_{max} 312 nm (ε5,000); pH 7 λ_{max} 294 (ε6,000), pH 12 λ_{max} 294 nm (ε6,200).

Synthesis of Poly(5-Dimethylaminocytidylic acid) [poly(m_2n^5C)] - A solution of m_2n^5CDP (30 mg) and polynucleotide phosphorylase (Micrococcus luteus 30 U/mg) (2.5 mg) in 0.2 M Tris-chloride (pH 9.0), 6.7 mM NaEDTA, 13.3 mM $MgCl_2$, 0.02% NaN_3, 2% BSA (5 ml) was incubated at 45° overnight. After deproteinisation, the aqueous phase was desalted by dialysis for 24 hours at 5° against 0.1 M NaCl, twice against 0.001 M NaEDTA and finally against water. Lyophilisation of the product at 0° gave poly(m_2n^5C) (13 mg), which ran as a single peak on polyacrylamide gel electrophoresis and had a $s_{20,w}$ of 8-12 as determined by ultracentrifugation in an isokinetic gradient of sucrose which contained acetate at pH 7.0. The ultraviolet spectrum of poly(m_2n^5C) at 20° in 0.1 M sodium acetate pH 7.0 was λ_{max} 294 ($\varepsilon(P)^6$ 5,700), 224 nm ($\varepsilon(P)$ 8,500). Spectrophotometric titration at 21° of poly(m_2n^5C) showed a sharp rise in the region of pH 4.1. Total hydrolysis of poly(m_2n^5C) by 0.1 N NaOH at 100° for 15 minutes gave a hyperchromicity in the uv spectrum of 4%. Hydrolysis of poly(m_2n^5C) (50 μM) by pancreatic RNase (0.5 μg) in 0.01 M ammonium acetate (0.2 ml, pH 7.0) at 25° gave the mononucleotide and was accompanied by a hyperchromic rise of 4% in the optical density at 272 nm; under these conditions (poly m_2n^5C) has $t_{\frac{1}{2}}$ = 2 min. and poly C has $t_{\frac{1}{2}}$ < 10 sec.

Preparation and Properties of a Poly(I) Poly(m_2n^5C) Hybrid - Equimolar quantities of poly(I) ($s_{20,w}$ = 6.64) and poly(m_2n^5C) were dissolved in 0.1 M potassium phosphate buffer pH 7.0 (2 ml) at 37° then cooled to 0°. After standing overnight the mixture was allowed to warm to room temperature and then applied to a Sepharose 4B 200 column (2 x 28 cm). Elution with water gave the hybrid poly(I) poly(m_2n^5C) in the void volume. The stoichiometry of hybridisation in 0.1 M potassium phosphate at pH 7 was determined by the method of continuous variations, monitoring the reaction at 245 nm. There was a sharp discontinuity in the curve at 50% molar concentration of poly(m_2n^5C) corresponding to the formation of a 1:1 hybrid. The melting temperature of poly(I) poly(m_2n^5C) in 0.1 M potassium phosphate pH 7.0 was 58°.

The position of substitution of the dimethylamine residue in m_2n^5C is established by NMR spectroscopy as the resonance due to H_5 is absent and the signal due to H_6 appears as a singlet at 7.6 ppm. Polymerisation of m_2n^5CDP by polynucleotide phosphorylase is slow and cannot readily be followed by phosphate release, prolonged incubation times being required for the production of poly(m_2n^5C). The polymer is more resistant to hydrolysis by pancreatic RNase than poly C and like poly C shows a sharp discontinuity around pH 4 on spectrophotometric titration, probably indicating the formation of an acid form of the polymer.

Poly(m_2n^5C) forms a 1:1 hybrid with poly(I) with a T_m of 58° in 0.1 M salt solution. This value is close to that for poly(I).poly(C) under comparable conditions and is lower than those values for hybrids of poly(I) with poly(5-halogenocytidylic acids)[7] or poly(m^5C)[8]. Thus there appears to be no simple relationship in the hybrids of poly(I) and poly (C) derivatives between the interstrand forces as measured by the T_m and any one parameter of the substituted cytidine nucleus e.g. pKa. The size of the substituent, its polarisability and its interaction with the solvent may all contribute to the interstrand stabilising forces. The activity of poly(I).poly(m_2n^5C) as an inducer of interferon is under investigation.

We thank the Nigerian Government for the award of a Federal Government Scholarship to J.O.F.

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POLY(5-FLUOROCYTIDYLIC ACID)

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(Received October 18th, 1973)

Summary

Poly(5-fluorocytidylic acid) [poly(f^5C)] has been prepared by the polymerisation of 5-fluorocytidine diphosphate with polynucleotide phosphorylase. The polymer forms a 1:1 hydrid with poly(I) and this hybrid is a good inducer of interferon. From a comparison of the melting temperatures of hybrids of poly(I) and poly(5-halogenated cytidylic acids) [poly(x^5C)], there appears to be a linear relationship between the T_m and the size of the substituent x.

Introduction

Hybrids formed between poly(I) and substituted poly(cytidylic acids) are of interest as potential inducers of interferon, and much work has been carried out to establish possible structure function relationships [1]. As a result of a report of a simple synthesis of 5-fluorocytidine [2], the preparation of f^5CDP, its polymerisation by polynucleotide phosphorylase to poly(f^5C), and the hybridisation of the latter with poly(I) appeared to be feasible. The preparation of poly(I)·poly(f^5C) would enable comparisons of the biological and chemical properties of this hybrid with those derived from the other poly(halogenated cytidylic acids).

Materials and Methods

Polynucleotide phosphorylase (polynucleotide orthophosphate:nucleotidyl transferase, EC 2.7.7.8) from *Micrococcus luteus* (30 units/mg) was purchased from Boehringer Corporation. *Crotalus adamanteus* venom and pancreatic ribonuclease was purchased from Sigma Corporation. Trifluoromethyl hypofluorite was obtained from Bristol Organics Ltd.

* To whom reprint requests should be sent.

5-fluorocytidine [2]. Trifluoromethyl hypofluorite (1.5 g) was passed into a solution of *N*,2′,3′,5′-O-tetraacetylcytidine [3] (5 g) in trichlorofluoromethane—chloroform (100 ml, 3:1, v/v) under nitrogen for 3 h at −80°C. After the solution had been flushed with nitrogen to remove excess CF_3OF, the solvent was removed in vacuo and the residue dissolved in methanol (80 ml). Triethylamine (20 ml) was added, the mixture heated under reflux for 30 min and then the solvent removed. Purification of the residue by ion exchange chromatography (Dowex 50 H^+, elution with dilute NH_4OH) gave f^5C which was recrystallised from water—ethanol—ether (1:50:50, v/v) to give 4.3 g (86%) as colourless needles, m.p. 222—226°C. Analysis found: C, 41.6; H, 4.77; N, 16.2; F, 7.03%. Calc. for $C_9H_{12}O_5N_3F$: C, 41.4; H, 4.63; N, 16.1; F, 7.2%. The ^1H NMR spectrum in ^2H_2O (220 MHz) showed H-6 as a doublet at τ 1.98 (J = 7.2 Hz) but no signal due to H-5.

5-fluorocytidine 5′-monophosphate was prepared from 2′,3′-*O*-isopropylidene f^5C (500 mg), prepared from f^5C by the method of Ueda [4], and trimethyl phosphate—phosphoryl chloride [5]. The chromatographically pure free acid was obtained by ion exchange chromatography using Dowex 50 (H^+ form) in 40% yield: ultraviolet spectra pH 2 λ_{max} 289 nm ϵ 6 600, pH 7 λ_{max} 279 nm ϵ 6 000, pH 12 λ_{max} 279 ϵ 6 200. Dephosphorylation of f^5CMP with *C. adamanteus* venom [7] gave f^5C as the only ultraviolet absorbing material.

5-fluorocytidine 5′-diphosphate was prepared as the trisodium salt in 64% yield from f^5CMP by the phosphoromorpholidate method [6]. Ultraviolet spectra (Fig. 1) pH 2 λ_{max} 290, ϵ 7 000; pH 7 λ_{max} 281 ϵ 6 500; pH 12 λ_{max} 280 ϵ 6 200.

Poly(f^5C). The polymerisation of f^5CDP was carried out as previously described for cl^5 CDP [8], the K_m and V for the reaction being 8.0 mM and 1.0 μmole/h, respectively. Poly(f^5C) which was obtained in 60% yield had an

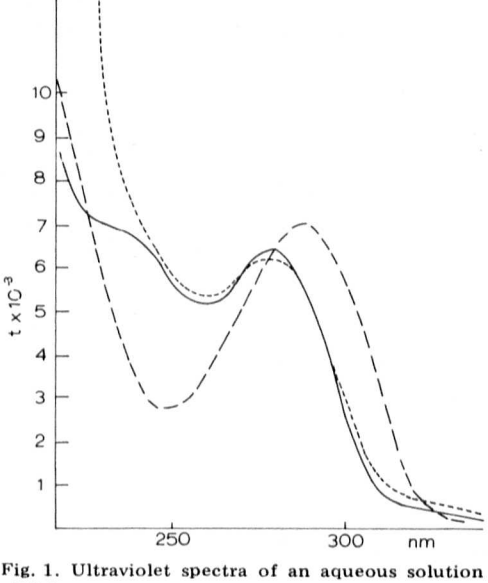

Fig. 1. Ultraviolet spectra of an aqueous solution of f^5CDP at 25°C. — — —, pH 2; ——, pH 7; - - - -, pH 12.

$s_{20,w}$ of 6.6 as determined by ultracentrifugation in an isokinetic gradient of sucrose containing 0.01 M potassium phosphate (pH 7.0), ultraviolet spectrum in 0.01 M sodium acetate (pH 7.0) λ_{max} 280 nm ϵ (P) 6200 [9]. Hydrolysis of a solution of poly(f^5C) in 0.1 M NaOH at 90°C for 15 min gave a hyperchromic rise in the ultraviolet spectrum of 14%, hydrolysis of a (0.2 μM) solution in 0.1 M sodium acetate (2 ml, pH 7.0) by pancreatic ribonuclease (0.5 μg) gave a hyperchromicity of 11%. The half life of poly(f^5C) under these conditions was 35 s, while the half life of poly(C) was 5 s. The CD spectrum of poly(f^5C) showed a maximum at 279 nm (θ = 32000) and a minimum at 216 nm (θ = 35000).

Preparation and properties of a poly(I)·(poly(f^5C) hybrid. Equimolar quantities as determined by ultraviolet spectroscopy, of poly(I) ($s_{20,w}$ 6.5) and poly(f^5C) were dissolved in 0.1 M phosphate buffer (2 ml, pH 7.0) at 37°C. After standing at room temperature for 2 h, the mixture was applied to a Sepharose-4B-200 column. Elution with water gave poly(I) · poly(f^5C) in the void volume. The stoichiometry of hybridisation in 0.1 M potassium phosphate at pH 7 was determined by the method of continuous variations [10], monitoring the hybridisation at 245 nm. There was a sharp discontinuity in the mixing curve at 50% molar concentration of poly(f^5C) indicating the formation of a 1:1 hybrid. The melting temperatures of poly(I)·poly(f^5C) in 0.1 M, 0.01 M salt solutions were 67 and 61°C, respectively.

Interferon induction with poly(I)·poly(f^5C). The hybrid (50 μg/ml) in phosphate-buffered saline was assayed for interferon induction by the inhibition of Semliki Forest virus nucleic acid synthesis in human embryo fibroblast cells [11]. The cells in Eagle's Minimum Medium (MEM) were incubated with the hybrid (50 μg/ml) in PBS, left for 1 h at 37°C, washed with medium (3×) and then covered with MEM containing 2% bovine serum. After standing overnight the supernatant was removed and assayed for interferon content by measuring the inhibition of [^{3}H]uridine uptake into trichloroacetic acid – insoluble material in human embryo fibroblast cells which had been challenged by virus. Poly(I)·poly(f^5C) produced 150 units of interferon under conditions when poly(I)·poly(C) produced 200 units.

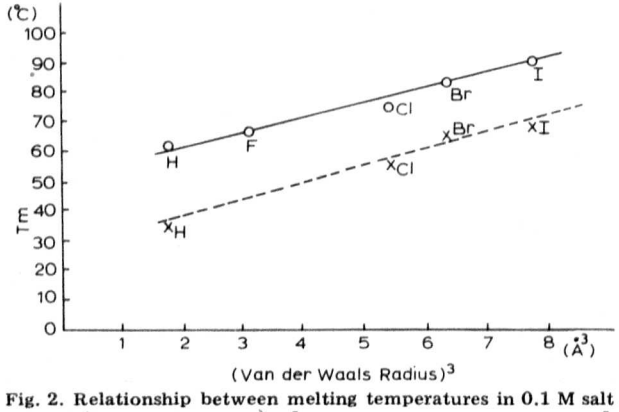

Fig. 2. Relationship between melting temperatures in 0.1 M salt solution and the size of the 5-substituent in the hybrids poly(rI)·poly(x^{5}rC) (——) and poly(dI)·poly(x^{5}rC) (– – –).

Discussion

Like other 5-halogenated cytidine diphosphates [8,12,13] f^{5}CDP is polymerised readily by polynucleotide phosphorylase to give a polynucleotide which possesses similar properties to poly(C) but is more resistant to hydrolysis by pancreatic ribonuclease. Poly(f^{5}C) forms a 1:1 hybrid with poly(I) which is a good inducer of interferon in human embryo fibroblasts. The melting temperature of the hybrid in 0.1 molar salt solution is 67°C which is between the values T_m of poly(I) · poly(C) and poly(I) · poly(cl^{5}C) [8] under these conditions.

It is of interest that the T_m values of the hybrids of poly(I)·poly(x^{5}C) varies in the series x = H, F, Cl, Br, I with the size of the substituent and not with its electronegativity. Indeed, there appears to be a straight line relationship between the T_m of the hydrid and the size of the substituent (as expressed by the cube of their van der Waals radii [14] (Fig. 2).

The sugar residue also plays an important part in stabilising the poly(I)·poly(C) hybrid as there is a considerable difference (26°C) in the T_m in 0.1 M salt of poly(rI)·poly(rC) and poly(dI)·poly(rC). We confirm this difference in a series of poly(dI)·poly(x^{5}rC) hybrids [15] but also observe that the T_m values of this series are directly proportional to the size of the substituent (Fig. 2). The hybrids poly(A)·2 poly(x^{5}U) form a series in which the T_m varies with the size of the halogen substituent [16,17]. However, the relationship in this case is not so clear probably because these hybrids are triple stranded. A few observations have been made on the T_m values of double stranded hybrids of poly(A)·poly(x^{5}U) but these are insufficient for firm conclusions to be reached. From a study of the properties of hybrids formed between poly(I) and poly(br^{5}C) containing varying amounts of bromine, Howard et al. [12] concluded that the polarisability of the bromocytidine was an important factor in stabilising the hybrid. Since the polarisability of an atom is proportional to its volume [18], the variation in T_m with the size of the halogen substituent which we find confirms this suggestion. Other substituents in the 5-position of the cytidine ring (e.g. Me- [19] or Me_2N- [20]) also stabilise the helix, the T_m values being 78 and 58°C, respectively. However, the stabilisation cannot be related directly to the size of the substituent in these cases probably because the relative complexity of these last two groups affects the relationship between size and polarisability. Furthermore, it has been shown [21] that the enthalpic stabilisation of a poly(I)·poly(C) hybrid by a 5-methyl group is less than that due to a 5-bromo atom.

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